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Phosphine fumigation and the ecology of the rust red flour beetle, *Tribolium castaneum*: the effects of phosphine resistance genes and sublethal exposure

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Abstract

Post-harvest losses of stored products are fairly common despite widespread use of chemical treatments to control pests. Phosphine has been used to control insect pests of various stored products in many parts of the world for at least half a century. However, extensive use of this fumigant has contributed to the development of resistance in several species of stored-product insects globally. Expression of phosphine resistance has been linked to mutations in two autosomal genes that contribute to enzymatic changes involved in basic metabolic pathways and impose metabolic stress, which, in turn, affects energy demanding behaviours.

Insecticide resistance is generally accompanied by a variety of physiological changes that may have negative influences on the organisms in the absence of the insecticide selective agent. The present investigations were undertaken to determine the effects of phosphine resistance genes on the fitness of *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) beetles, serious pests of stored products worldwide. I designed laboratory assays to examine if the phosphine resistance genes affect activities that depend on a reliable energy source, including particular aspects of the beetle's walking and flight activities. I measured walking and flight parameters of four *T. castaneum* genotypes: 1) a field-derived population, 2) a laboratory cultured, phosphine-susceptible reference strain, 3) a laboratory cultured, phosphine-resistant reference strain, and 4) a resistant introgressed strain that is almost identical genetically to the susceptible population. The temporal pattern of flight was identical across all populations, but resistant reference beetles took flight significantly less, walked more slowly, and located resources less successfully than did susceptible reference beetles. These results suggest an association between phosphine resistance and the reduced ability of these resistant reference individuals, and they suggest that these influences are likely to have an impact on the spread of the resistance genes spatially.

I also conducted a series of experiments in a wind tunnel to determine whether intrinsic variables (mating status) and environmental variables (presence or absence of lures, type of lures) further influence the movement abilities of the phosphine resistant beetles. Significantly fewer flight initiations and less success at locating pheromone and food lures by resistant reference beetles were observed compared with those of the susceptible reference ones (although their odour preferences were similar). Pheromone and cotton seeds appeared to be stronger inducements for location of the lure compared with wheat flour. A clear

preference for the pheromone lure was evident in unmated resistant beetles compared to mated resistant beetles. However, this pattern was not repeated with flying resistant beetles, as no significant difference was observed in flight response of mated and unmated resistant beetles towards the pheromone lure. Mating status had statistically similar effects on the movement behaviours of susceptible reference beetles. These results show that phosphine resistance genes lead to slower movement of the beetles relative to susceptible reference beetles, accompanied by a significantly lesser ability to locate sources of food and pheromones.

The flight responses and resource location behaviours of resistant and susceptible beetles were tested prior to and after exposure to a sublethal dose of phosphine, using the methods developed for the experiments described above. At the same time, the metabolic rate of resistant and susceptible individuals was also measured, and all measurements were made before and after exposure to a sublethal dose of phosphine. An understanding of how the survivors of sublethal exposures behave is important for predicting the spread and local build-up of phosphine resistance genes. The activity levels of beetles and their metabolic rate were negatively correlated with the presence of resistant genes. Populations with resistant genes showed significantly lower levels of activity in both flight and walking and also lower metabolic rates. After treatment with sublethal doses of phosphine (LC_{10}), beetles of all strains showed a reduction in metabolic rate and also in flight and walking activities. Reductions in metabolic rate and movement activity were significantly stronger in susceptible reference beetles. The relatively lower reductions in metabolic rates and locomotory activities of the resistant reference insects exposed to a sublethal dose of phosphine are predicted to enhance the spread of resistance alleles spatially among populations and increase the flow of resistance genes among them.

The overall results of this study lead to two general conclusions. First, all the results derived from the comparisons of the performance of field and laboratory beetles in this study showed that the field beetles walked significantly faster and more directly towards food resources and had a higher propensity for flight, lower metabolic rate and lighter body mass than the laboratory beetles. These effects of the laboratory culturing of beetles warrant serious consideration in future studies on these organisms, as interpretations could be affected by the culturing history of the organisms tested. Second, this study demonstrated the significant effects of phosphine resistance genes and sublethal exposure of phosphine on the metabolic

rate and movement responses of these insects. These effects need to be investigated in the field, because altered rates of migration by resistant beetles, in association with sublethal exposure to phosphine, may have to be considered seriously when designing phosphine resistance management programs.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-reviewed papers

Malekpour, R., Rafter, M.A., Daglish, G.J. & Walter, G.H. (2016) Influence of phosphine resistance genes on flight propensity and resource location in *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae): the landscape for selection. Biological Journal of the Linnean Society, DOI: 10.1111/bij.12817.

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Chapter 2

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Contributor	Statement of contribution
Malekpour, R.	Designed experiments (70 %) Data analysis (90 %) Wrote the paper (70 %)
Rafter, M.A.	Designed experiments (10 %) Data analysis (10 %) Wrote the paper (10 %)
Daglish, G.J.	Designed experiments (10 %) Wrote the paper (10 %)
Walter, G.H.	Designed experiments (10 %) Wrote the paper (10 %)

Contributions by others to the thesis

Significant contributions were made by Professor Gimme H. Walter, Dr. Gregory J. Daglish and Dr. Michelle A. Rafter to the conception and design of the project, as well as providing useful advices in terms of analysis and interpretation of research data.

Pieter Arnold, who works with another research group in the School of Biological Sciences, contributed advice on the metabolic rate measurements (a part of the results of Chapter 4), and provided training with respirometry assessments.

A joint paper has been accepted by an international peer reviewed journal on a topic related to the results presented in this thesis. It was envisaged to be part of a collaboration with Pieter Arnold and that work would have amalgamated our respective methodologies into an evolutionary study related to beetle migration, but the collapse of cultures prevented these plans being put in place (see Appendix).

Statement of parts of the thesis submitted to qualify for the award of another degree

None

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Keywords

Tribolium castaneum, laboratory culturing, phosphine resistance, sublethal fumigation, flight propensity, resource location, metabolic rate, natural selection.

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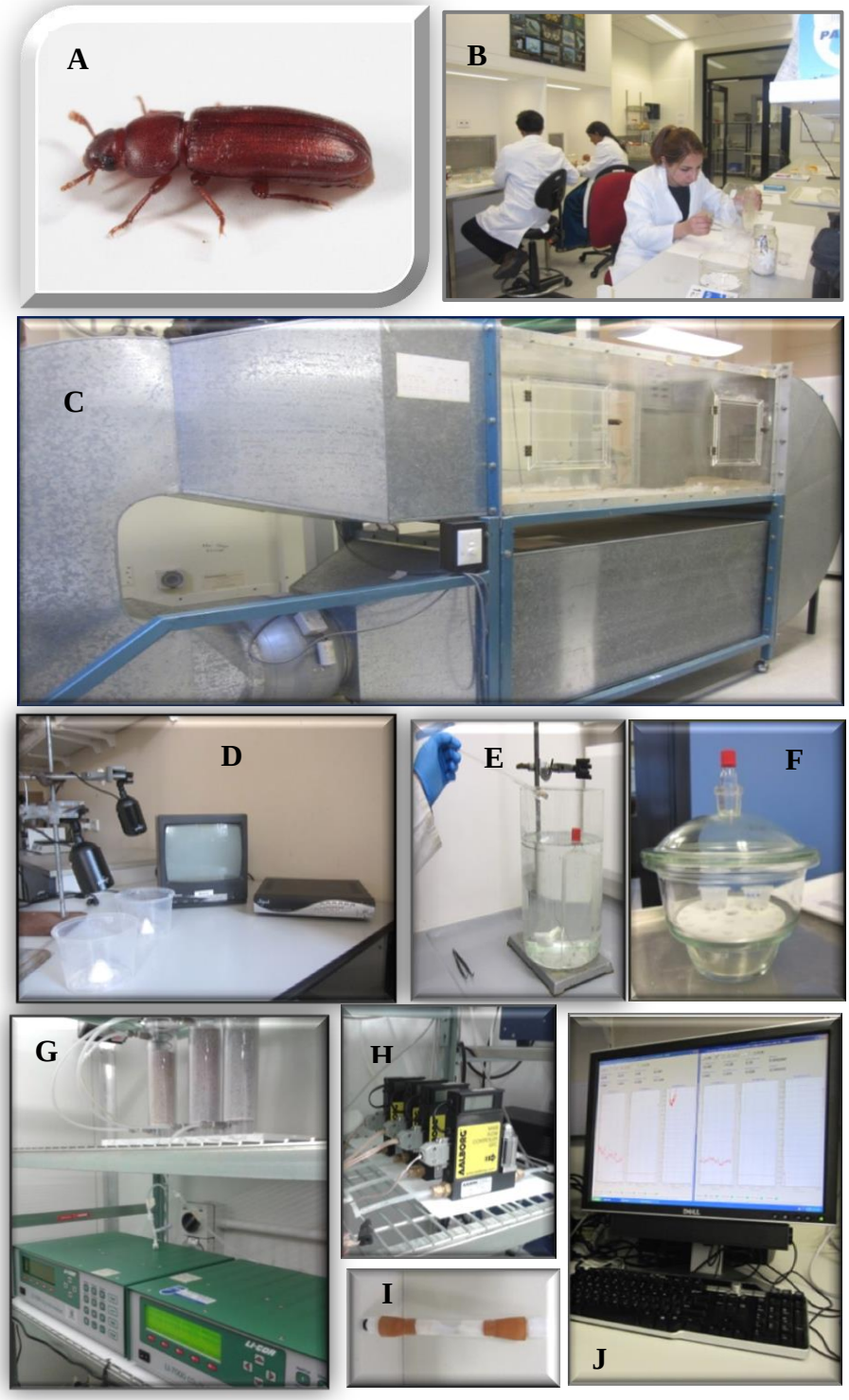

Frontispiece.

A. Adult *Tribolium castaneum* **B.** Laboratory culturing of the beetles **C.** Wind tunnel to assay food location and flight propensities **D.** Flight chambers to assay flight periodicity **E.** Phosphine generation apparatus **F.** Fumigation chamber **G to J.** Respirometry setup.

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List of Abbreviations used in the thesis

PH_3	Hydrogen phosphide
FAO	Food and Agriculture Organization
DLD	DihydroLipoamide Dehydrogenase
ELISA	Enzyme-Linked-Immunosorbent Serologic Assay
QTC4	Queensland *Tribolium castaneum* 4
QTC931	Queensland *Tribolium castaneum* 931
ISOTC24	Isogenic *Tribolium castaneum*
PC	Personal Computer
DNA	DeoxyriboNucleic Acid
PCR	Polymerase Chain Reaction
GLMM	General Linear Mixed Model
GLM	Generalized Linear Model
df	Degrees of Freedom
RH	Relative humidity
ANOVA	Analysis of Variance
LED	Light Emitting Diode

Chapter 1

General Introduction

1.1. Stored grain insect pests- dispersal mechanism

The management of insect pests that infest post-harvest products has always received less attention than efforts to manage the pests of field crops, despite the losses they cause. Harvested grains may be stored, in bulk, for varying periods in storage facilities prior to their consumption or trade. During the period of storage, a number of insect pest species may attack bulk stored grains and cause substantial damage to these products (Hagstrum et al., 1999, Hagstrum and Subramanyam, 2006). Recent estimates put global post-harvest losses of grains at nearly 120—160 million tons per year, or 10-15 percent of all stored grain globally (Boxall, 2001, Rajendran, 2002). Insects are among the most successful groups of organisms that attack stored products and contibute substantially to post-harvest losses of grains and grain quality (Pimental, 1991). The orders of insects associated with stored products are Hymenoptera, Hemiptera, Psocoptera, Lepidoptera and Coleoptera (Reed, 2006, Rees, 2004). The last two are the major groups of economically important insect pests, with a greater diversity of beetles than caterpillars attacking stored products (Rees, 2004).

A basic aspect in the life of most organisms is to disperse (Mazzi and Dorn, 2012). This behaviour plays an important role in population persistence, population dynamics and the distribution of insect pests (Shivankar et al., 2006). The dispersal of insect pests is a topic of great concern in developing integrated pest management programs, and is a central concern in pest management in field crops.

Dispersal may be defined as a form of movement that contribute to gene exchange (Ronce, 2007). Some studies have attempted to relate genetic variation in natural systems to the movement of organisms as an integral part of their life-history (Roderick, 1996, Mazzi and Dorn, 2012). The individual movements into or out of resource patches results in the

connection of local populations of a species and leads to the movement of alleles from one population to another one (Smouse et al., 2010). Fundamentally, daily and seasonal movements between localities occur by means of one or two distinct processes. Passive dispersal includes movements that are not under the direct control of the organisms and are largely governed by external factors such as physical vectors (like air and water currents) or biotic vectors (including human activities) (Matthysen, 2012). Active dispersal involves movements that are controlled by the organisms themselves and are dependent on various aspects of their internal condition (including genetic and physiological features) in relation to environmental cues and the suitability of localities (Matthysen, 2012). Active dispersal is regarded as a three-stage process that comprises the leaving of the locality (emigration), a phase of active movement (the "vagrant phase"), and the detection of a new locality and settling there (immigration) (Matthysen, 2012; Ronce, 2007, Walter, 2003). Evolutionary interpretations suggest that a complex set of environmental variables and a number of individual characteristics affect the three stages of active dispersal (Bowler and Benton, 2005, Cox and Collins, 2002), and the little that is known in this regard about insect pests of stored products is summarized in Table 1.1.

Table 1.1. Examples of environmental and individual factors known to influence the propensity for dispersal by insect pests.

Species	Factor		Movement response	Literature source
	Environmental	Individual		
Anthonomus grandis		Life stage	Lower flight activity in females with fully mature eggs	Rankin et al., 1994
Calopteryx splendens		Sex	Higher dispersal rate in female adults	Chaput-Bardy et al., 2010
Tetraopes tetraophthalmus		Sex	Higher dispersal rate in male adults	Lawrence, 1988
Tribolium castaneum		Age	Lower flight activity in aged adults	Perez-Mendoza et al., 2011a; b
Rhyzopertha dominica	Light		Greater flight activity at dusk	Perez-Mendoza et al., 1999
Sitophilus oryzae	Temperature		No flight initiation below 27.5˚C	Cox et al., 2007
Prostephanus truncates	Density		Higher flight initiation in cultures of high density	Fadamiro et al., 1996
Tribolium castaneum	Resource quality		More beetles leave diets of lower-nutritional value	Fedina and Lewis, 2007
	Light intensity, relative humidity, temperature		Flight initiation did not vary with light intensity and relative humidity. However, the temperature and photoperiod affected their flight initiation.	Perez-Mendoza et al., 2014
Lasioderma serricorne	Temperature	Sex, age and mating status	The minimum flight initiation temperature was between 19-22°C. Young virgin males and older virgin females had the highest flight initiation among all categories.	Fardisi and Mason 2013

1.2. Strategy of management of stored grain insect pests

Because food is susceptible to attack along its entire processing chain, pest populations must be monitored at different stages along the chain, including at on-farm storage systems, warehouses, food processing equipment and packing houses (Reed, 2006). The efficiency of grain quality maintenance in storage facilities depends, to a considerable extent, on the management tactics employed against stored grain pests, and these can be managed by applying various combinations of non-chemical and chemical control options (Campbell et al., 2004). Standard non-chemical management strategies include sanitation, aeration (making the grain cooler than the initial storage temperature), grain drying, biological control (using natural enemies of the pest to reduce pest populations) and surveillance for pest insect infestations (Campbell et al., 2004, Rajendran, 2005, Reed, 2006). Various methods are used to inspect for insect infestations in storage facilities, including visual inspection, sampling and sieving, flotation methods, fragment counts, staining techniques, CO_2 analysis, uric acid determination and imaging techniques (Rajendran, 2005, Reed, 2006). Some studies have typically recommended that using extreme temperatures can be an effective non-chemical method for controlling the insect pests of stored grain (Fields, 1992, Loganathan et al., 2011).

Chemical options usually are the most prominent part of most stored food pest management programs. They can be broadly categorized into contact insecticides and fumigants. Most contact insecticides, applied as sprays and dusts, have long-lasting residual action (Matthews, 2008, Penrose et al., 1994, Reed, 2006). Their residues provide longer action against pests, but are unacceptable on food (Reed, 2006). Fumigants, by contrast, dissipate from foodstuffs to the air with minimal chemical residues and they are applied as solids or gases (Reed, 2006). Therefore fumigation of stored grain is the preferred method of chemical control. A number of alternative fumigants have been used, including hydrogen cyanide, sulfuryl

fluoride, methyl iodide, methyl bromide, carbonyl sulphide and ethyl formate (Bell, 2000, Fields and White, 2002, Ren and Mahon, 2007, Zettler and Arthur, 2000). Before 2005, methyl bromide and phosphine were the only two licensed fumigants used world-wide against stored grain pests. Methyl bromide's classification as an ozone-depleting substance under the Montreal Protocol means its use has been restricted globally (Carter et al., 2005). With only one ideal fumigant remaining, pest management programs now rely almost entirely on phosphine as the preferred chemical control tactic (Pike, 1994).

Phosphine, hydrogen phosphide (PH_3), is a colourless, odourless and flammable toxic gas (Chaudhry, 2000). It has been used globally for over half a century to disinfest insect pests in a range of stored products (Chaudhry, 2000). Its continuous and widespread use is desirable because of its relatively cheap cost, ease of generation and application, rapid diffusion through bulk-stored grains and rapid decomposition into environmentally safe compounds (Nayak and Collins, 2008). Furthermore, phosphine does not affect the viability of seeds and can be used in relation to a wide range of commodities (Nayak and Collins, 2008).

1.3. Biochemical and physiological aspects of phosphine toxicity

Although the exact mode of toxic action of phosphine is not fully understood, studies have shown that phosphine toxicity causes various biochemical changes and physiological responses in different animals. Three related postulates of phosphine's toxic action have been recorded, namely neural, metabolic and redox (Nath et al., 2011). The neural aspect of phosphine toxicity is associated with an inhibitory effect on acetylcholine esterase (an enzyme that helps regulate acetylcholine signalling) and causes excessive acetylcholine signalling (Al-Hakkak et al., 1989). Overactivity of acetylcholine signalling occurs immediately following phosphine exposure, with consequent hyperactive responses (Winks,

1985). The metabolic response to phosphine toxicity is related to the inhibition of cellular respiration (Price and Dance, 1983). Phosphine interacts with complex IV, cytochrome c oxidase of the mitochondrial respiratory chain and disrupts ATP synthesis, and this could lead to death (Chefurka et al., 1976). Finally, phosphine may contribute to cellular oxidative stress due to its inducing reactive oxygen species (Chaudhry and Price, 1992). Inhibition of cytochrome c oxidase by interaction of phosphine with complex IV may lead to inadequate electron transfer to molecular oxygen and induce the generation of reactive oxygen species (Mráček et al., 2009). Reactive oxygen species, including superoxide and hydrogen peroxide, have a high potential ability to damage macromolecules and may thus lead to cell death (Chaudhry and Price, 1992). In spite of the documented differences in physiology across vertebrates and invertebrates, the essential features of phosphine toxicity are similar for all animals and the modes of action are not mutually exclusive (Bond et al., 1969, Proudfoot, 2009). Phosphine is considered to be a respiratory poison because it produces toxic oxy-radicals and disrupts the oxygen metabolism of animals that have been exposed to the gas (Chaudhry and Price, 1992). This point is expanded further below.

1.4. Principles of phosphine activity

The toxicity of a typical fumigant generally results from its concentration (C) × exposure time (T) and this is the equation that expresses the dosage of the fumigant (Hole et al., 1976). The mode of action of phosphine differs from that of other fumigants in that longer exposures to phosphine of relatively lower doses are more effective than shorter exposures at higher concentrations of the gas (Chaudhry and Price, 1990a; Hole et al., 1976). Indeed, high concentrations of phosphine induce narcosis in stored-product insects (and presumably other organisms) (Nath et al., 2011), a physiological state that may protect insects from the toxic effects of the phosphine gas (Hole et al., 1976). Extended exposure periods should be

considered the main strategy in achieving maximum effectiveness of phosphine against pests (Benhalima et al., 2004).

Since phosphine typically occurs in gaseous form, its main entry into the exposed animal is through the respiratory system. Therefore, it is believed, any change in the respiration rate may affect the uptake of this fumigant. For example, a drop in temperature is reported to diminish the toxicity of phosphine to the organism through decreasing its metabolic rate and the consequent lower uptake of the fumigant (Chaudhry et al., 2004). The toxicity of phosphine to insect pests is enhanced by a rise in the level of carbon dioxide because this increases the rate of respiration, and results in a higher uptake of phosphine (Kashi and Bond, 1975). Since the rate of respiration of adult insects is different from the respiratory rate of the pre-adult stages, it is believed that the rate of phosphine uptake differs across the different stages of life (Howe, 1973). The most tolerant stages to phosphine were anticipated to be the egg and pupal stages, as their respective metabolic rates were the lowest (Bell, 1976, Howe, 1973, Lindgren and Vincent, 1966).

1.5. Outcomes of sublethal exposure to phosphine

For efficient fumigation, storage facilities need to be air-tight (sealed) to ensure commodities are fumigated at the correct concentration for the entire duration of fumigation (Benhalima et al., 2004). This is often not the case in storage facilities across many countries (Boxall, 2001). Gas leaks from facilities that should be air-tight expose the insects to sub-lethal doses of the fumigant, which in turn reduces the efficacy of the fumigation. Such conditions lead to an increase in the number of fumigations required to control insect pests, and consequently allows those phosphine resistant insects that would normally be killed by the fumigation to

survive. Poor fumigation thus inadvertently increases the frequency of resistant individuals in the population.

a) Effect on physiological processes

Insect pests have evolved a range of behavioural responses under sublethal phosphine exposure (Hobbs and Bond, 1989, Ridley et al., 2012). These behavioural responses minimize exposure to the fumigant and are reported to be protective mechanisms that can lead to insect survival (Guedes et al., 2008). The fecundity and offspring production of females that survive a sublethal fumigation are reduced compared to unfumigated females (Ridley et al., 2012). In addition, temporary delays in the hatching of eggs of *Tribolium. castaneum* (Herbst, 1797) females exposed to sublethal doses of phosphine have been reported (Rajendran, 2000). Moreover, sublethal exposure to phosphine reduced walking activity, an indication of lower metabolism rates in the fumigated insects, which also contributes to a minimization in the uptake of phosphine (Pimentel et al., 2012).

b) Development of phosphine resistance

The lack of cheap residue-free alternatives to phosphine for use as a fumigant against different pests in grain storage has resulted in the widespread and frequent application of this chemical (Benhalima et al., 2004, Opit et al., 2012). Moreover, fumigation of storage facilities where phosphine gas is rapidly lost due to poorly sealed structures results in lower levels of phosphine than those required to kill the pests (Benhalima et al., 2004). Repeated inadequate fumigation therefore has led to the development of phosphine resistance and this threatens the reliability of this fumigant. Detection of phosphine resistance was first reported by the Food and Agriculture Organization (FAO) survey of 1972-1973, which showed that nearly 10% of the populations collected from different countries were resistant to phosphine

(Champ and Dyte, 1976). Since then, much higher levels of resistance have been recorded globally and this situation becomes a major problem in many countries.

Phosphine resistance in various pests of stored products has developed across countries of different socio-economic status, including Brazil (Athié et al., 1998, Lorini et al., 2007, Pimentel et al., 2010), the United States of America (Opit et al., 2012, Zettler and Cuperus, 1990), Australia (Kaur et al., 2012, Nakakita and Winks, 1981) and Pakistan (Alam et al., 1991). At least 11 species of stored grain pests have proved resistant to phosphine, including *Rhyzopertha dominica* (Fabricius, 1792), *Sitophilus oryzae* (Linnaeus 1763), *T. castaneum*, *T. confusum* (Jacquelin du Val, 1863), *Cryptolestes ferrugineus* (Stephens 1831), *Trogoderma granarium* (Everts, 1898), *Lasioderma serricorne* (Fabricius, 1792) and *Oryzaephilus surinamensis* (Linnaeus, 1758) (Ahmedani et al., 2007; Bengston et al., 1999, Daglish et al., 2014, Jagadeesan et al., 2016; Lorini et al., 2007; Pimentel et al., 2007, Rajendran and Narasimhan, 1994, Zettler and Cuperus, 1990).

1.6. Genetics of phosphine resistance

Detailed genetic analyses of *T. castaneum* (Jagadeesan et al., 2012), *R. dominica* (Schlipalius et al., 2002), *S. oryzae* (Nguyen et al., 2015) and *C. ferrugineus* (Jagadeesan et al, 2016) have identified two major autosomal genes that are linked to the expression of phosphine resistance, but which are incompletely recessive. These genes, *rph1* and *rph2*, result in two distinct phenotypes that are well known from resistance screening bioassays, and are called the ‘weak’ and ‘strong’ resistance phenotypes (Collins et al., 2002). Molecular genetic analysis has demonstrated that each single autosomal gene, on its own, is responsible for weak resistance but the synergistic interaction between the *rph1* and *rph2* genes provides the strong resistance phenotype (Jagadeesan et al., 2012, Schlipalius et al., 2008). Genetic

experiments in *R. dominica* suggested the possibility of a third gene contributing to strong phosphine resistance in these pest insects (Collins et al., 2002), but that has not been resolved. This additional dominant gene has been found in *C. ferrugineus* beetles, and this has additive effects on the level of phosphine resistance (Jagadeesan et al, 2016). However, this additional factor has been observed neither in *T. castaneum* (Jagadeesan et al., 2012) nor in *S. oryzae* (Nguyen et al., 2015).

1.7. Behavioural changes associated with phosphine resistance

Selection for resistance to phosphine, as an evolutionary process, is often assumed to be accompanied by changes in the fitness of the organism in the absence of phosphine exposure (Daglish et al., 2015, Pimentel et al., 2007), which are referred to as "fitness costs". Such costs generally result from allocating the energy of a basic biological process to the protective behavioural mechanisms that work against the activity of the insecticide, and this may lead to the impaired performance of that particular biological process (Groeters et al., 1994). Two general approaches have been applied to the study of the fitness costs associated with selection for resistance, namely the population cage approach and physiological performance approach (Daglish et al., 2014, Pimentel et al., 2007). Population cage studies are conducted with a hybridized population that results from the crossing of susceptible and resistant strains. This population is then cultured in the absence of phosphine selection for multiple generations, when the frequencies of the resistant phenotype and/ or resistant genes are measured (Jagadeesan et al., 2012). A study based on this approach showed the frequency of the resistance genotype in *R. dominica* remained unchanged across 20 generations, which indicates the absence of fitness costs associated with phosphine resistance (Schlipalius et al., 2008).

The second approach examines a range of physiological parameters in relation to different levels of resistance. A study, derived from this approach, assessed the respiration and population growth rates of *T. castaneum, O. surinamensis* and *R. dominica* with different levels of resistance (Pimentel et al., 2007). The populations that showed lower respiration and growth rates were those with the higher resistance ratios, which suggest the existence of physiological fitness costs in resistant populations (Pimentel et al., 2007). Clarification of the degree of fitness costs associated with phosphine will provide new sights on the course of evolution of phosphine resistance and should help to improve resistance management strategies.

1.8. The rust-red flour beetle, *Tribolium castaneum*

Tribolium castaneum is a worldwide pest of great economic significance, causing considerable loss of stored products (Mundakkal et al., 2011). It is one of the most common tropical storage pests, best known for infesting flour and milled cereals (Boon and Ho, 1988, Campbell and Arbogast, 2004, Campbell et al., 2010). *Tribolium castaneum* is a small beetle (2.3 - 4.4 mm in length) reddish brown in colour, and commonly referred to as the "rust-red flour beetle" or the "red flour beetle". Its severity as a pest of stored products is related to particular aspects of its life history, namely long life span for such a small insect, short life cycle, the ability of rapid development and the ability to start reproduction a few days after eclosion and to continue laying eggs throughout its long adult life (Dawson, 1977).

Tribolium castaneum individuals readily move between multiple food patches, and pheromone trapping data from the field (coupled with population genetic analyses) indicate these insects may well fly distances of a hundreds kilometres and perhaps more (Ridley et al., 2011). However, the rate of dispersal is relatively low in immature adults, peaks at or after

the start of reproduction, and starts to decline gradually with increasing age (Perez-Mendoza et al., 2011a, Ziegler, 1976). Besides adult age, some other factors are known to influence the dispersal of these insects, including temperature, humidity and light (Speight et al., 2008), temperature gradients (Jian et al., 2005), quality and quantity of food (Fedina and Lewis, 2007, Perez-Mendoza et al., 2011a), and volatiles emitted by grains and their associated fungi (Ahmad et al., 2012). Beetles deprived of food for a short period had a higher tendency to initiate flight compared with those having access to food (Perez-Mendoza et al., 2011a, b). The longer the duration of starvation, the lower the rate of flight initiation, but prolonged starvation (about 18 days) resulted in complete inhibition of flight initiation (Perez-Mendoza et al., 2011a; b). The investigation of the movement ecology of *T. castaneum* as a stored grain pest leaves a number of unanswered questions, although it does provide a sound basis for further studies of pests that carry phosphine resistance genes.

1.9. Objectives and thesis structure

The central aim of the study presented in this thesis is to contribute to our understanding of the potential effects of phosphine resistance genes on the fitness of *T. castaneum* beetles, especially in relation to individual movement. This study also investigated whether such effects on fitness could be induced by sublethal phosphine exposure.

Chapter 2 presents data that derive from laboratory experiments on particular aspects of the walking and flight activities of laboratory and field-cultured populations of *T. castaneum* in association with their phosphine resistance status. Of special importance here was the inclusion of a resistant introgressed strain that had been developed previously from repeated backcrossing of both resistant and susceptible strains followed by phosphine selection on the

progeny from each backcrossing, to produce a resistant strain that shares much of the genetic background of the susceptible reference strain.

Chapter 3 examines, with the aid of a wind tunnel, the relative performance of resistant and susceptible beetles to various odours, to establish whether environmental factors (volatiles associated with different resources) and intrinsic factors (mating status and resistance status) induce variation in the walking and flight responses of resistant beetles relative to those of susceptible population.

Chapter 4 tests the physiological effects of sublethal exposure to phosphine on resistant and susceptible beetles (including those from the resistant introgressed strain). The specific objective of this chapter is to examine whether sublethal exposure to phosphine impacts on the metabolic rate and movement propensity of resistant and susceptible individuals. A better understanding of the direction of these behavioural changes that follow sublethal exposure to phosphine, could help in improving phosphine resistance management programs and then help to reduce the frequency of resistance in the field.

Chapter 2

Influence of phosphine resistance status on flight propensity and resource location in *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) - the field for selection

This results chapter has been published in a peer reviewed journal, and is edited slightly according to the format of other results chapter of the thesis.

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The co-authors of this paper are my thesis supervisors, Prof. Gimme Walter, Dr. Gregory Daglish and Dr. Michelle Rafter. They provided feedback on the experimental design, analysis and interpretation of research data, and manuscript drafts. My contribution to the published paper involved initial concept and experimental design, data collection, data analysis and preparation of the manuscript.

2.1. Introduction

The evolution of insecticide resistance has become an ever-present challenge to food security and health worldwide. Influential factors in the evolution of resistance to insecticides include the mode of inheritance of resistance, the initial frequency of resistance alleles in populations, and the life-history traits of the organisms (Collins et al., 2002, Lockwood et al., 1984, McKenzie 1996, Taylor, 1986, Zettler et al., 1989). Further, the frequency of migration and the movement abilities of the species concerned are of primary importance in the spread of resistant populations (Pasteur & Raymond, 1996, Pimentel et al., 2010). Differences in the movement abilities of individual organisms of a species, from being relatively sedentary to highly active (Gurarie et al., 2010, Ritte & Lavie, 1977), are assumed to be underpinned by genetic influences (Gurarie et al., 2010, Korona, 1991, Meylan et al., 2009, Ritte & Lavie, 1977). If genes actually influence the propensity of organisms to migrate from a locality, does it mean that this behaviour is under direct genetic control or do genes affect other mechanisms (such as metabolic pathways), and thus influence movement indirectly? Studies on the movement of resistant insects are therefore central to understanding the evolution, spread and persistence of insecticide resistance alleles in populations, and in the spatio-temporal dynamics of that resistance. Nevertheless, the evolution of resistance has generally received little attention from this perspective and, particularly so, the potential influence of pleiotropic effects on the spatiotemporal dynamics of insecticide resistance. This is the aspect explored through the laboratory research presented here.

Tribolium castaneum (Herbst), the red flour beetle, is a worldwide pest of great economic significance because it causes considerable loss of stored grains (Campbell et al., 2004, Herron, 1990), and is best known for infesting flour and milled cereals (Boon & Ho, 1988; Campbell & Arbogast, 2004; Campbell et al., 2010). Individuals of this species readily move

between multiple food patches, by walking and by flight (Perez-Mendoza et al., 2011a; Ridley et al., 2011), so they are evidently well adapted to spatially heterogeneous landscapes. Phosphine has long been the leading disinfestation option for stored-grain insects (Chaudhry, 1997). An increased dependence on this fumigant as the control method of choice is attributable to the low residue levels left, its fast diffusion in air, and low cost (Chaudhry, 1997; Nayak et al., 2013). Also, it is suitable for use on a wide range of commodities and in a range of storage situations (Ahmedani et al., 2007; Nayak & Collins, 2008). The long history of phosphine use (since the 1970s) and its often poor application have contributed to selection of phosphine resistance in a range of storage pests in many countries (Lorini et al., 2007, Opit et al., 2012, Daglish et al., 2015). Detailed genetic analysis has identified two major autosomal genes that are linked to the expression of phosphine resistance, but which are incompletely recessive (Jagadeesan et al., 2012). Both *rph1* and *rph2* confer weak resistance on their own, but the synergistic interaction of these alleles results in strong resistance to phosphine (Jagadeesan et al., 2012, Schlipalius et al., 2008).

Resistance to phosphine is mediated by a metabolic enzyme, dihydrolipoamide dehydrogenase (DLD), which is an essential component of four major metabolic multienzyme complexes in mitochondria (Schlipalius et al., 2012). Mutations in the DLD gene, representing the *rph2* allele, are directly responsible for phosphine resistance and result in substitutions to amino acids associated with the disulfide catalytic centre in the DLD protein (Schlipalius et al., 2012). These changes reduce the electron transfer rate in mitochondria and thus affect various basic metabolic pathways (Chaudhry, 1997).

Resistance to phosphine therefore has the potential to affect a range of physiological processes that would typically incur fitness costs (Daglish et al., 2014), and indications are

supportive to some extent. Resistant *T. castaneum* females have been recorded laying fewer eggs than susceptible ones (Saxena & Bhatia, 1980). Both reproduction and respiration rates among resistant populations of *T. castaneum* were lower than in susceptible ones (Pimentel et al., 2007), and such populations had lower developmental and population growth rates than susceptible populations (Sousa et al., 2009). The consequences of any fitness disadvantages imposed on those individuals with resistance to phosphine are, therefore, potentially significant influences with respect to the spatio-temporal dynamics of phosphine resistance. Some studies, using the population cage approach, show no evidence of any fitness cost associated with weak resistance (Daglish et al., 2015), and others suggest that resistance alleles do not affect the mobility of insects (Kaur et al., 2013). Clarification of the degree to which phosphine resistance alleles impose fitness costs should enhance the prospects for improving resistance management strategies (Lockwood et al., 1984).

We therefore designed laboratory assays to examine if the phosphine resistance status of *T. castaneum* individuals affects activities that depend on a reliable energy source, including particular aspects of their walking and flight activities. An understanding of these influences should be significant to interpreting the geographical spread and local build-up of phosphine resistance genes (Fragoso et al., 2005; Oliveira et al., 2007).

2.2. Materials and methods

2.2.1. Insect culture

Three laboratory strains and a field cultured strain of *T. castaneum* were used for behavioural assessments. For the field strain, beetles were collected from a farm storage in Warwick, Queensland (28° 13′ 0″ S and 152° 1′ 0″ E), two months before the start of each bioassay. One hundred unsexed field-collected beetles were placed in a 500 ml glass jar containing 200

g of rearing medium made up of 95 parts wheat flour to 5 parts powdered yeast. After 7 days, all founder adults were removed by sieving the medium so as to obtain a culture of similar-aged larvae. The adults were transferred to a new culture jar to continue oviposition. Pupae of each culture were sexed and kept individually within wells of ELISA plates until the emergence of adults. After emergence, the adults were maintained individually in plastic cups with rearing diet for 2 weeks, so that the age and nutritional status of individuals used in assays were similar. The rearing cycle of these "field beetles" was repeated weekly for 6 weeks to produce enough first generation field adults for testing. Cultures were maintained at 25˚C and 46% r.h. and 12:12 L: D photoperiod.

Three laboratory strains of *T. castaneum*, QTC4, QTC931 and ISOTC24, are maintained at the Department of Agriculture and Fisheries in Brisbane, Queensland, Australia. The QTC4 strain originated from a storage facility in Brisbane in southern Queensland in 1965 and has been cultured ever since in the absence of selective pressure from phosphine exposure. These insects are susceptible to phosphine and are referred to as the "susceptible reference strain" throughout this manuscript. The QTC931 strain, referred to as the "resistant reference strain", was established with phosphine resistant individuals collected from Dalby, southern Queensland in 2000 and is about 400× more resistant to phosphine than the susceptible strain (Jagadeesan et al., 2012). A "resistant introgressed strain" (ISOTC24) was derived from both the resistant and susceptible strains by a process of repeated backcrossing of resistant virgin males to susceptible virgin females followed by phosphine selection on the progeny from each backcrossing, to produce a resistant strain that shares much of the genetic background of the susceptible reference strain. The resulting strain is 300× more resistant than the susceptible strain, and 98.4% genetically similar to the susceptible strain based on six

backcrosses. To obtain beetles of similar age from these different strains, they were cultured in the same way as described above for the field beetles.

2.2.2. Flight bioassay

a. Periodicity of flight

To film and assess the time of day during which flight initiation peaks, flat-bottomed cylindrical polypropylene plastic containers (8 cm height × 10 cm diameter) were made into small flight chambers (Fig. 2.1A). The inner walls of all vertical surfaces were covered with Fluon® (AGC Chemicals Americans, Inc., Exton) to prevent the insects from walking up the walls of the flight chambers. To facilitate flight initiation, a truncated filter-paper cone with a hole cut at its peak was placed in the centre of each flight chamber. A transparent lid was put on the container to prevent the flying insects from leaving the container. Each individual adult was placed on the base of the container and immediately covered with a plastic cup, in which it was allowed to acclimate for 2 hours. The cup was then removed, so the insect could move freely and its behaviour recorded by video camera (Signet, model No QV-3020, Capalaba, Australia). The beetle took off from the paper cone and fell when it hit the lid, a process that occurred several times during the observation period, which started at 14:00 h each day and ended at the same time the following day. During each 24-hour period of observation, the behaviour of four individual adults (one in each of four containers) was recorded by each of four video cameras. PC Viewer software (Stentofon Baudisch, Waeschenbeuren, Germany) allowed each individual beetle to be tracked (on video) throughout the observation period and was used to count the number of flight take-offs of each beetle over a 24-hour period as the insect flew from the cone. The experiment was conducted with 20 individuals of each sex and of each strain at 25˚C on sunny days, in a laboratory with windows, to use natural light as the only light source. Experiments with field

beetles were run during June and July 2013, and the other strains were tested during June and July 2014.

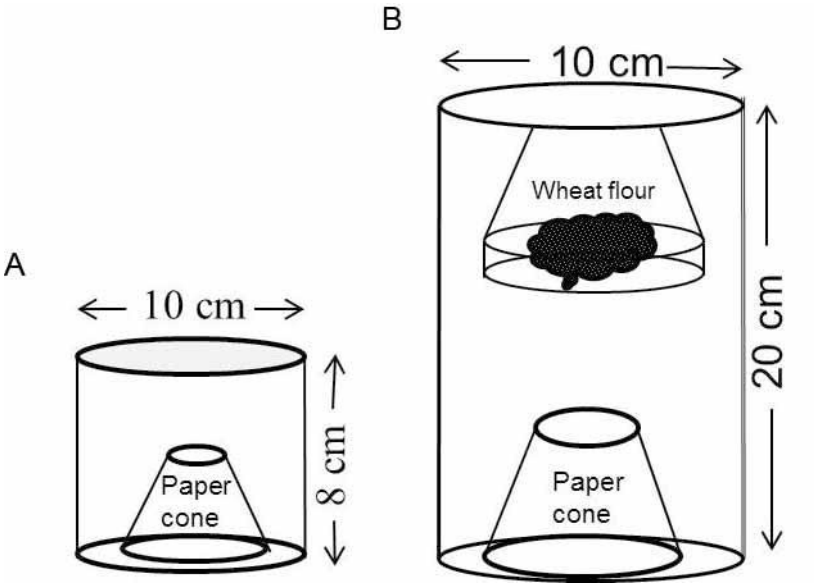

Fig. 2.1. Schematic diagrams of the chambers used to examine flight periodicity (A) and flight propensity (B) of *Tribolium castaneum* adults.

b. Measurement of flight propensity

To measure flight propensity, a taller flight chamber was constructed from a perspex cylinder (20 cm height × 10 cm diameter) (Fig. 2.1B). A flat filter paper platform, baited with 50 g of wheat flour, was suspended with string from the top of the flight chamber and a truncated cone platform was placed on the floor of the chamber. The inner walls of the flight chamber and the bottom of the suspended bait platform were coated with a thin layer of Tangle-trap® sticky compound (Contech Enterprises Inc., Victoria, Canada) to trap any flying beetles when they made contact with a surface. The flight propensity of the field strain and three laboratory strains was evaluated during September 2014 in a controlled environment room at 26˚C and 60% r.h. Each treatment was conducted with 40 beetles (20 of each sex). Each round of tests

was conducted with four flight chambers, and groups of 20 adults of the same sex were placed on the floor of each cylinder, and on each day each of the four strains was represented. Ultimately five replicates were run, each of 20 beetles of each sex and of each strain. Flight activity was most intense in the late afternoon (see Results), so tests were begun 2 hours preceding sunset and continued for 3 hours each day. The numbers of beetles adhering to the sticky surfaces were counted at the conclusion of each test period.

2.2.3. Walking bioassay

The walking activity of all strains of *T. castaneum* was bioassayed between 10:00 and 15:00 h (a time when they are usually not flying) in a rectangular perspex wind tunnel (120 cm length × 60 cm width × 50 cm height) at 26˚C, 60% r.h. and ambient light conditions, during August and September 2014. All daily assessments were run with individual beetles of different sexes and different strains (n= 15 for each sex of each strain). The push–pull airflow wind tunnel was surrounded by black curtains. Wind was pushed through the tunnel by a variable speed fan set at 1.5 m/s. At the start of each experiment, wind speed was checked using a Testo 405-V1 anemometer (Instrument Choice, Adelaide, Australia) at the centre of the wind tunnel and a height of 5cm. The walking bioassay was conducted with 30 adults introduced individually in the centre of the wind tunnel. A petri dish containing 250 g of clean wheat flour was placed upwind, 50 cm from the release point of the beetles. A video camera, held on a tripod, was positioned above the wind tunnel to record the walking tracks of each insect for 20 min. The numbers of beetles locating the food source or walking in any direction that ultimately took them away from the food source were noted. Several variables associated with the movement of the insects, including velocity (cm/s), angular velocity (rad/s) (as a measure of the complexity of the pathway taken by the beetles) and distance

moved (cm), were calculated with the aid of video tracking software (EthoVision, Noldus Information Technology, Wageningen, The Netherlands).

2.2.4. Screening for strong-resistance genes in field beetles

To determine whether any of the field collected beetles in our study were strongly phosphine resistant, we sequenced a region of the DLD gene containing the specific point mutation, previously identified as contributing to the strongly resistant phenotype of *T. castaneum* (Schlipalius et al., 2012).

An 800-bp region of the DLD gene was amplified using the primers DLD530F (TGCAATCGGCCATTCGAAAC) and DLD1422R (ACAAAGTTGGCACCACTACCT). PCRs were carried out as 12 µl reactions containing 1X My Taq (Bioline) buffer, 0.2 µM each of forward and reverse primer, 0.3 units of Taq polymerase and 2 µl of template DNA. PCR cycling conditions consisted of an initial denaturing step of 95°C for 2 min, followed by 35 cycles of 95°C for 25 sec, 57°C for 30 sec and 72°C for 1 min, with a final extension step of 72°C for 10 min. PCR products were cleaned with ExoSAP (Glenn & Schable, 2005), and then sequencing reactions performed using a capillary ABI3730 Genetic Analyser (Applied Biosystems). Sequences were aligned by eye in Geneious 7.1.6 (Kearse et al. 2012), and compared with sequences from the resistant reference strain and the susceptible reference strain downloaded from GenBank to assess the presence of the specific G to A mutation associated with the strongly resistant phenotype.

2.2.5. Statistical analysis of walking and flight data

For flight data, the effects of light, sex and phosphine resistance status on flight periodicity of beetles were tested by fitting a generalized linear mixed effects model with a Poisson

distribution family (GLMM), which is appropriate for heterogeneous count data (Wedel et al., 1993). The data from the flight propensity tests were analysed using a generalized linear model (GLM) with a binomial distribution family. Subsequent post hoc pairwise differences were determined among treatments using Tukey's test.

For walking data, we compared the number of field and different laboratory strain individuals that reached the food in the wind tunnel with a Pearson's chi-square test. Also, we applied a generalized linear model (GLM) with Gaussian distribution to determine how sex, phosphine resistance status, and extended laboratory culturing were associated with the walking parameters. Interactions between sex and treatments were statistically insignificant, and so were not included in the analyses presented. Multiple comparisons (Tukey's post hoc test) were also performed to examine if specific pairwise comparisons between treatments were significantly different. All analyses were performed using the "RStudio" statistical program, RStudio (R Development Core Team, 2013).

2.3. Results

2.3.1. Flight bioassay

a. Periodicity of flight

The flight periodicity of beetles from the field and laboratory strains is shown in Fig. 2.2. Although some flight was observed early in the day, flight activity peaked at the end of photophase and into the beginning of scotophase. It then declined to the lowest level, almost no activity at all, at dawn. Light significantly affected the degree of flight activity of different strains of laboratory-reared beetles and also field ones (GLMM, $Z = 8.1$, $P < 0.001$ and GLMM, $Z = 3.7$, $P = 0.0002$ respectively), with the temporal pattern of flight being similar across the different strains (Fig. 2.2). However, the susceptible reference strain beetles took

off for flight significantly more frequently than did resistant reference strain beetles (GLMM, Z = -23.1, $P < 0.001$) and field strain beetles took off much more frequently than did those from any of the other strains tested (Fig. 2.2). Statistical comparison is not permissible because there was a several-month gap between the flight test of field strain beetles and the flight test of other strains. The flight periodicity pattern of adult males was statistically similar to that of females for the different strains of laboratory-reared beetles, and also for the field strain **(**GLMM, Z = 0.8, P = 0.3 and GLMM, Z = 1.1, P = 0.2, respectively). Therefore, the data from males and females were combined in the statistical tests.

b. Flight propensity

No significant differences were evident between males and females, in any treatment, in their flight propensity (GLMM, Z = -0.98, P = 0.3). The flight propensity of the field strain beetles differed, however, from that of all three strains of laboratory beetles, namely the resistant reference strain (GLMM, Z = -12.9, $P < 0.001$), resistant introgressed strain (GLMM, Z = -13.15, $P < 0.001$) and susceptible reference strain (GLMM, Z = -9.4, $P < 0.001$) (Table 2.1). Subsequent post hoc analyses (Table 2.1) demonstrate no significant differences between the number of flights initiated by beetles in the resistant reference strain and resistant introgressed line beetles, but there were significant differences between these resistant strains compared with each of the susceptible strains (both laboratory and field beetles) (Table 2.1).

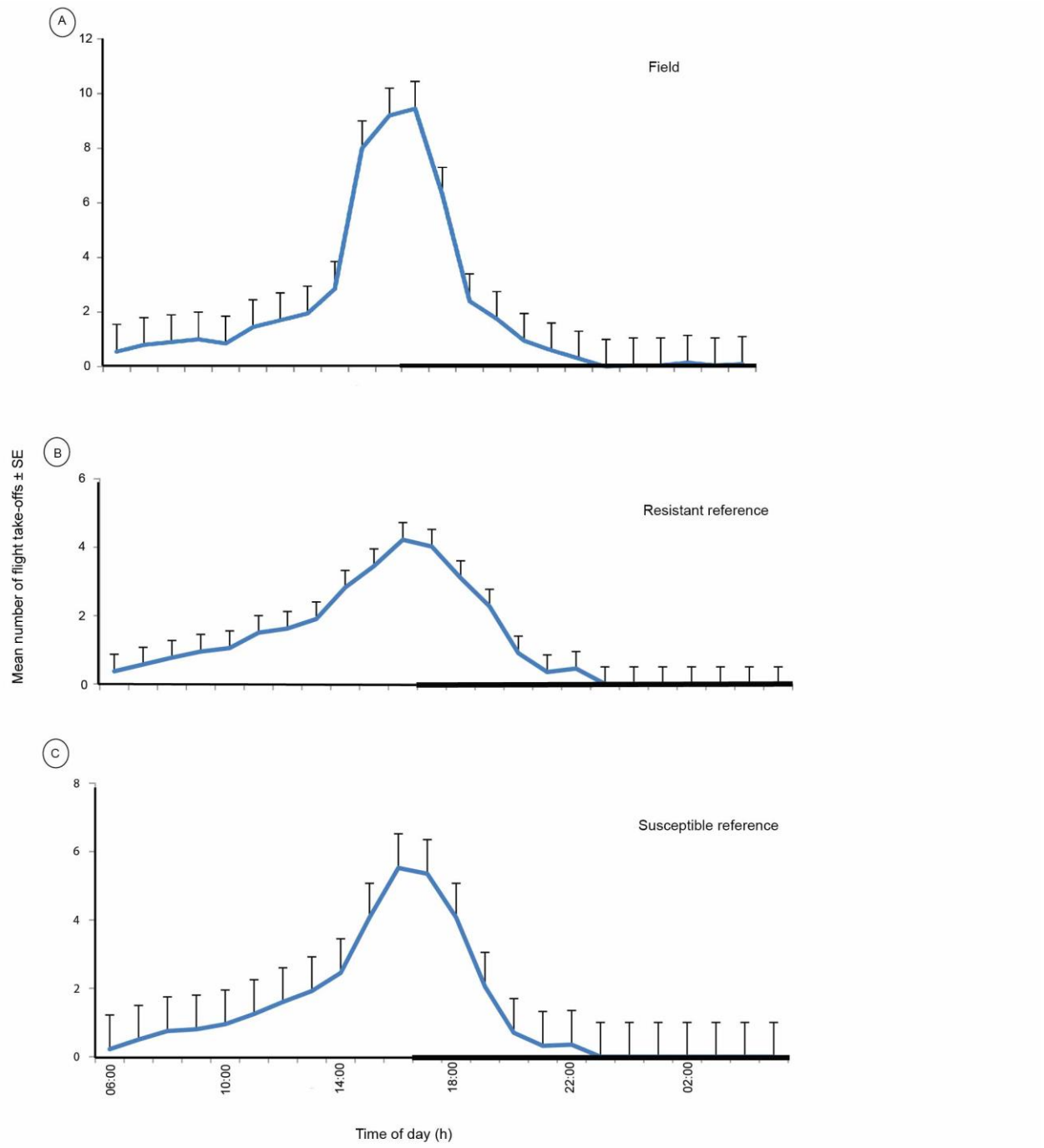

Fig. 2.2. Daily flight activity patterns of *Tribolium castaneum* adults that derive from a phosphine susceptible reference strain (bottom), phosphine resistant reference strain (middle) and from field individuals (which are phosphine susceptible) (top), in relation to time of day. Dark lines on the x-axis indicate the dark phase of the experiment (during which only natural light was used), n = 20 for each sex of each strain.

2.3.2. Walking bioassay

The number of beetles that located food is given in Table 2.2 for each strain. The highest percentage of beetles that located the resource within 20 min was in the field beetle treatment (83%) (n =30), and significantly fewer beetles from the resistant laboratory strains did so (40-47%) (Chi-square test on the raw data representing field vs resistant introgressed strains ($\chi 2$ = 7.32, df = 1, P = 0.006) and field vs resistant reference strains ($\chi 2$ = 10.1, df = 1, P = 0.001). The susceptible reference strain did not differ statistically from the field strain ($\chi 2$ = 2.1, df = 1, P = 0.1), but with only 63% finding the resource.

The variables measured to represent the responses of beetles to resources whilst walking included velocity, distance moved (between the release point and the food resource) and angular velocity. These parameters all varied significantly across the different strains (Fig. 2.3). Field beetles moved significantly faster than beetles of all three laboratory strains (field vs resistant reference strains, GLM, T = -7.8, P < 0.001; field vs resistant introgressed reference strains, GLM, T = -6.5, P < 0.001; field vs susceptible reference strains, GLM, T = -3.2, P < 0.01) (Fig. 2.3A). However, the total distance that field beetles moved was significantly less than the distance moved by beetles from the other laboratory strains, whether resistant reference (GLM, T = 9.5, P < 0.001), resistant introgressed reference (GLM, T = 6.4, P < 0.001) or susceptible reference strains (GLM, T = 2.7, P < 0.001) (Fig. 2.3B). Field beetles showed the lowest degree of angular velocity among all treatments and this was significantly different compared with resistant reference strain beetles (GLM, T = 2.7, P = 0.006), but not statistically different from that of the resistant introgressed strain beetles (GLM, T = 1.3, P = 0.1) and susceptible reference beetles (GLM, T = 0.3, P = 0.7) (Fig. 2.3C).

Table 2.1. Mean (± SE) flight propensity of field strain and different laboratory strain individuals of *Tribolium castaneum* in relation to sex. No significant differences were detected between the sexes in any treatment (GLM, see text for details), and field strain beetles differed significantly in flight propensity relative to all other strains (as indicated by different letters in the table, which are based on Tukey's post hoc pairwise comparisons). Number of replicates = 5 per sex of each strain, 20 beetles in each replicate.

Treatments	Number of trapped beetles		Statistical differences
	Female	Male	
Field strain	18.6 ± 0.01	18.4 ± 0.01	a
Resistant reference strain	2.8 ± 0.01	2.4 ± 0.02	b
Resistant introgressed strain	2.2 ± 0.01	2.0 ± 0.01	b
Susceptible reference strain	8.8 ± 0.03	7.6 ± 0.02	c

The walking speed of susceptible reference beetles was significantly higher than the speed of walking of resistant reference and resistant introgressed strain beetles, whereas the walking speed of resistant reference beetles was not significantly different from that of resistant introgressed strain beetles (Fig. 2.3A). The total distance moved by susceptible reference beetles was statistically less than the distance moved by resistant reference and resistant introgressed strain beetles (Fig. 2.3B). Also, there was a significant, but weak difference in the total distance moved by resistant reference beetles relative to resistant introgressed strain beetles (Fig. 2.3B). No significant difference was detected between the angular velocity of walking of laboratory susceptible reference and resistant introgressed strain beetles, but the lowest angular velocity was recorded for susceptible reference beetles (Fig. 2.3C). A Tukey's post hoc pairwise comparison showed no significant difference in the rate of angular velocity

between resistant reference and resistant introgressed beetles, which indicates a similar rate of turning across these two strains (Tukey's test, $Z = -0.15$, $P = 0.99$).

Table 2.2. Total numbers of field and different laboratory strain individuals of *Tribolium castaneum* that reached a distant food resource by walking (n=30 for each strain). Those beetles that walked in directions other than that in which the food was placed all failed to locate the food resource. See text for results of statistical comparisons. The significant statistical differences in the numbers of beetles that successfully found the food have been indicated by different letters (Chi-square test).

Treatments	Number and percentage of beetles reaching food	Statistical differences
Field strain	25 (83%)	a
Resistant reference strain	12 (40%)	b
Resistant introgressed strain	14 (47%)	b
Susceptible reference strain	19 (63%)	ab

2.3.3. Screening for strong-resistance genes in field beetles

Sequencing an 800-bp region of the DLD gene from 70 field-collected beetles yielded eight discrete haplotypes (GenBank accession numbers KU679900-KU679907). When aligned with the phosphine resistant strain none of the sequences was found to contain the specific G to A point mutation that typifies the resistant strain, indicating that none of the field beetles was strongly resistant to phosphine.

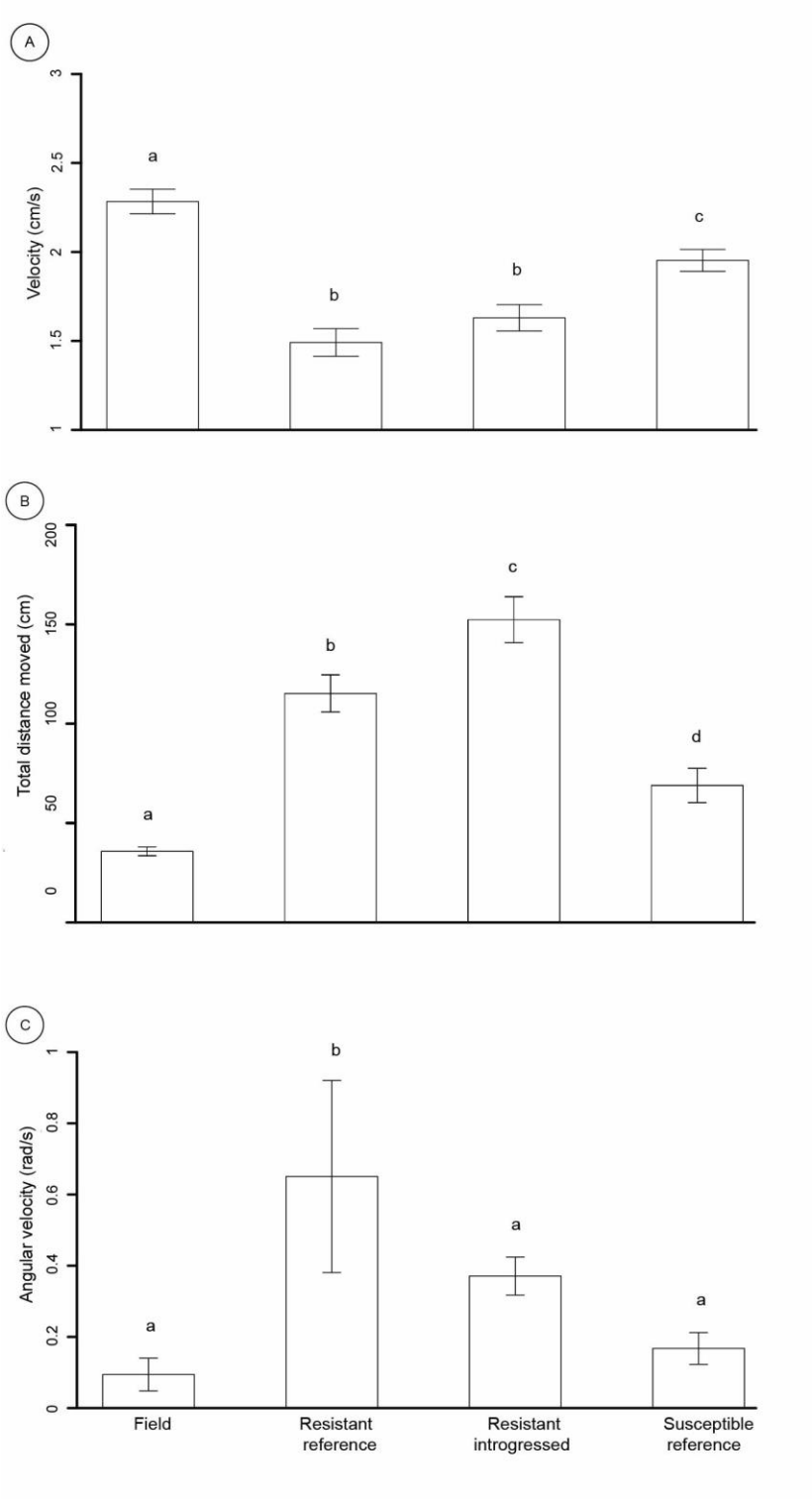

Fig. 2.3. Various measures of the movement of *Tribolium castaneum* adults from a field and three laboratory strains with different degrees of resistance to phosphine fumigant, n = 15 for each sex of each strain. (A) Velocity (cm/s), (B) distance moved (cm) and (C) angular velocity (degree/s). Different letters indicate significant differences among strains ($P < 0.05$) (Tukey's post hoc test).

2.4. Discussion

Our investigation probed the way in which the genetic control of phosphine resistance affects aspects of the movement of *T. castaneum*, for this could influence the rate and distance components in the spread of phosphine resistance genes in the field. In other words, we could assess the likelihood of the selection pressures faced by beetles that carry the alleles for resistance extending well beyond the sites at which phosphine is applied.

Our experimental work, conducted with laboratory susceptible reference and resistant reference strains, also included a resistant introgressed strain that is genetically very similar to the susceptible reference strain we used, besides carrying the resistant alleles. The importance of the resistant introgressed strain is that any differences observed between the movement abilities and propensities of susceptible reference and resistant reference strain beetles would likely be related to the presence of the phosphine resistance gene. Indeed, the introgression approach is recognised as an important means of controlling for genetic background when comparing susceptible and resistant strains (e.g. Raymond et al., 2011).

The pattern of flight periodicity of both sexes of beetles from the two resistant strains we tested coincided closely with that of both field and laboratory susceptible reference beetles. The peak time of flight take-off was at dusk (17:00 h) irrespective of the presence of phosphine resistance alleles. These results are similar to those that Boon & Ho (1988) obtained for *T. castaneum* in a rice warehouse in Singapore, for they observed a rapid increase in trap catches after 14:00 h which then reached a peak at 18:00 h. We also demonstrated a negative correlation between the propensity for flight take-off and the presence of phosphine resistance alleles. That is, the susceptible reference strain beetles took

to flight significantly more frequently than did beetles in either of the resistant strains we tested (Fig. 2.2).

The walking velocity of the resistant reference strain as well as that of the resistant introgressed strain was significantly lower than that of beetles from both susceptible strains (field and laboratory). These findings with respect to beetles carrying phosphine resistance genes, together with the findings of Pimentel et al. (2007) on the lower respiration rates of resistant beetles, support the hypothesis we tested that genetic changes associated with phosphine resistance alleles (which affect basic metabolic pathways) will, in turn, influence negatively any behavioural activities reliant on those metabolic enzymes, including flight and walking. To determine whether respiration was indeed sub-optimal in our phosphine resistant strains, we measured metabolic rates in all four strains we tested and these results were consistent with those of Pimentel et al. (2007) (Malekpour, unpublished data). Such differences in locomotory and respiratory responses of insecticide-resistant populations compared with susceptible ones have been demonstrated in other stored-product pests (Oliveira et al., 2007, Pimentel et al., 2012) and not only for conventional insecticides (Correa et al., 2015).

Not only did the resistance allele affect the rate at which resistant beetles walked, but the total distance walked and the angular velocity of walking were lower in both susceptible strains than in those individuals carrying phosphine resistance alleles (Fig. 2.2). That is, the susceptible beetles walked more directly to the resource, whereas resistant beetles wandered around more slowly over a significantly longer distance, and fewer of them actually located the resource during a 20-min assay period (Table 2.2). These results thus imply that pleiotropic effects of the phosphine resistance alleles may affect the orientation abilities of

the insects, and this confirms that the area in which resistance alleles are exposed to selection in the field extends to all areas beyond the immediate sites of phosphine application. Future studies on fitness costs associated with phosphine resistance should focus on testing the sensitivity of the olfactory receptors and should perhaps screen the chemicals perceived by the antennal receptors of phosphine resistant individuals relative to those of phosphine susceptible ones.

Our results also add to the growing body of evidence as to the ways in which the culturing of insects inadvertently selects behaviour that is significantly different from that of their wild type conspecifics. Wild population *T. castaneum* beetles have been shown to have much higher fecundity (Ahmad et al., 2012) and shorter development time (White, 1984) relative to laboratory beetles. Also, ground-up cotton seed supported the development of field population *T. castaneum* larvae to some extent, whereas laboratory population larvae could not survive on this particular resource (Ahmad et al., 2012). By contrast, the pupae of laboratory population beetles also weigh more than those of field population beetles (Toro et al., 1979; White, 1984). In our case, the differences in movement propensities and characteristics between field and laboratory strains of *T. castaneum* (besides the differences attributed to the phosphine resistance alleles) could be related to the continued easy access to high quality food resources for beetles over many generations in the laboratory culture, without any need to disperse and locate resources. That is, the usual selection on the foraging behaviour of individuals experienced in nature could be inadvertently relaxed in culture, with these behavioural mechanisms then being eroded through pleiotropic effects as unintentional selection on other features is imposed. Also, the laboratory strain was weakly active in both flight and walking behaviour compared to the beetles reared as first generation offspring from field collected adults. Field individuals, for example, walked more directly towards food and

had to cover significantly less distance to reach the food relative to laboratory beetles (Figs. 2.2B and 2.2C).

The results we have presented indicate an association between phosphine resistance and the reduced ability of these phosphine resistant individuals to move effectively in relation to their ecological requirements. The influence of these alleles on the locomotory activity of the insects carrying them will, therefore, influence the spread of resistance alleles spatially among populations. In particular, their lower rates of movement and less directed orientation relative to distant resources could reduce gene flow among populations relative to the rate that would be predicted from observations on the behaviour of susceptible insects. That is, selection on resistant individuals away from the sites of phosphine treatment is likely to work against the establishment of resistance in entirely susceptible populations. Possibly, trade in commodities could be a more important contributor to the expansion of resistant populations than movement of individuals carrying phosphine resistance alleles, but this requires direct test in the field. We also wonder whether the lower activity levels of resistant individuals could lead to reduced phosphine exposure and thus, in turn, reduce fumigation efficacy. These potential impacts of the resistance alleles need to be understood so that resistance mechanisms can be included more accurately and effectively in resistance management strategies. Such understanding would also sharpen our perspective on how the pleiotropic effects of new mutations on the behavioural patterns of organisms might influence the fate of those alleles in the broader landscape across which organisms move and settle.

Chapter 3

Initiating emigration and influencing resource location- environmental and intrinsic variables interact in phosphine resistant *Tribolium castaneum* beetles

3.1. Introduction

The movement of individuals across heterogeneous landscapes on a daily, seasonal or even irregular basis is a fundamental feature of the ecology of virtually all species (Hirao et al., 2008, Nathan et al., 2008, Walter & Hengeveld, 2014). Successful movement between areas of suitable habitat is essential for their survival and reproduction, and impacts the spatio-temporal dynamics of the species (Ronce 2007, Walter & Hengeveld, 2014). Naturally, ecological studies have found significant variation in the movement of organisms of particular species and in the factors driving this variation (Borgemeister et al. 1997, Edde et al. 2006). Understanding the underlying basis of this movement has proved elusive. In some studies, emigration rates correlated with density (Fadamiro et al., 1996), but in many more they do not (Perez-Mendoza et al., 2011). This investigation therefore focuses on a system in which a genetic influence on propensity to fly has been demonstrated recently, and in which the same genes influence the ability of the insects to localize suitable resources (Malekpour et al., 2016), as explained next.

The rust-red flour beetle, *Tribolium castaneum* Herbst, is a major cosmopolitan pest and appears to be pre-adapted to feeding on stored grains, especially milled cereal products, stored flour and fungus-infested grain (Via 1991, Campbell & Runnion 2003, Hagstrum & Subramanyam 2006). Despite the reputation of these insects for being generally flightless, they do leave bulk grain storages and fly substantial distances across the landscape, which results in samples of these beetles being genetically homogenous over thousands of square kilometers (Ridley et al., 2011). They evidently move readily among widely dispersed resources, because flying beetles can detect volatile stimuli that originate from stored food resources and move towards them (Ahmad et al., 2013). This well-developed ability to move among multiple food patches, even by walking (Campbell & Hagstrum, 2002), contributes to

their rapid infestation of nearby established resources in the form of stored grains (Perez-Mendoza, Campbell & Throne 2011a). Further, the aggregation pheromones produced by males that have already colonised a resource increase the attractancy of that particular food resource (Phillips et al. 1993).

Despite the colonization abilities of *T. castaneum* beetles, as outlined above, their propensity to fly and their flight characteristics are little known. Some factors involved in *T. castaneum* taking flight have been examined, for example, temperature (Cox et al., 2007), population density (Perez-Mendoza et al. 2011a, b) and aspects of the physiological state of the insects (Fedina and Lewis 2007, Perez-Mendoza et al., 2011a, b), Also, the influence of wind direction in the field on the orientation of their flight, which is largely downwind, has been quantified, and it is clear they do not fly when wind speed is too high (> ~ 3 m/s) (Rafter et al., 2015). The role of genes in emigration of the beetles by flight, and the colonization of new resources remains unclear, but the previous study showed that genes associated with resistance to phosphine are associated with a reduced propensity to fly, as well as a decrease in velocity and more turning whilst walking towards a food resource (Malekpour et al., 2016). These features have consequences for the behaviour associated with beetles leaving an environment that is still of good quality for them, and the subsequent colonization of new resources. These are the issues explored through the laboratory research presented here. Specifically, I investigated how the impaired movement abilities of beetles, which result from the pleiotropic effects of their phosphine resistant status, affect the process of their initiating flight and locating new resources.

Do attractive volatiles provide an external inducement factor that increases the relatively lower rate of movement of those beeetles that carry the phosphine resistant genes? Does the

mating status of beetles carrying phosphine resistant genes affect their tendency to fly in the same way as it does for susceptible beetles? This study employed short-range bioassays, in a wind tunnel, to provide information on the role of sex and mating status on the resource location abilities of phosphine-resistant adults of *T. castaneum* relative to these features in susceptible beetles from a long-term laboratory colony (as well as insects from a recently established (one generation) field colony). The ultimate goal was to establish whether genetic influences on the exploratory movement of beetles would override environmental influences of the factors affecting movement, by exploiting the understanding of how the phosphine resistance alleles affect movement of these insects. This study thus provides quantified insights into the propensity of organisms to leave suitable resources and move on. I also demonstrate how the flight of *T. castaneum* beetles and their abilities to locate resources are influenced by the genes that code for phosphine resistance.

3.2. Materials and methods

3.2.1. Insect strains and culturing

The *T. castaneum* adults used in this study originated from three laboratory strains and a culture established with field-collected beetles. These latter insects are referred to as "field beetles" and these were tested within one generation of their being brought into the laboratory. Two months prior to each set of assessments, *T. castaneum* beetles were collected from farm storages near Dalby (27° 11′ 0″ S and 151° 16′ 0″ E), west of Brisbane, Queensland, Australia. Each such culture was initiated with 100 adults and these were allowed to oviposit on 200 g of organic wheat flour supplemented with powdered yeast (5%). The founder beetles were transferred to a new culture jar after 7 days to continue mating and oviposition. First generation pupae from these field-collected beetles were sieved from each culture jar, sexed and housed individually within wells of an ELISA plate until their

emergence as adults. The young emerged adults were fed on wheat flour for 2 weeks before the start of the experiments, to ensure the nutritional status of the sexed adults was similar. This standardization controlled for potential differences in movement behaviour that might have resulted from age and nutritional status. The rearing cycle was repeated every week over an 8-week period to produce enough first-generation adults. All cultures were maintained at 25˚C, 46% RH and 12:12 L:D photoperiod. Culturing methods for the laboratory strains were similar to those described above for the field beetles.

The laboratory strains of *T. castaneum* used in this study included QTC4 (susceptible reference), QTC931 (resistant reference) and ISOTC24 (resistant introgressed). The strains were obtained from cultures held at the Department of Agriculture and Fisheries in Brisbane, Queensland, Australia. The susceptible reference strain derived from a storage facility in Brisbane, southeast of Queensland in 1965 and has been cultured in the laboratory, since then in the absence of selective pressure from insecticides (Jagadeesan et al. 2012). The phosphine resistant reference strain originated from a resistant field sample collected from Dalby, southern Queensland, in 2000 and is about 400× more resistant to phosphine than the susceptible reference strain (Jagadeesan et al. 2012). The resistant introgressed strain was developed from both the resistant reference and susceptible reference strains by a process of repeated backcrossing of resistant reference virgin males to susceptible reference virgin females, followed by phosphine selection on the progeny from each backcrossing, to produce a resistant reference strain that shares much of the genetic background of the susceptible reference strain. The resulting strain is 300× more resistant than the susceptible reference strain, and 98.4% genetically similar to the susceptible reference strain based on six backcrosses (Daglish, unpublished data). The importance of the resistant introgressed strain is that any differences observed between the movement abilities of susceptible reference and

resistant reference strain beetles would likely be related to the presence of the phosphine resistance gene. To obtain beetles of similar age from these different strains, they were cultured in the same way as described above for the field beetles.

3.2.2. Odour stimulants and mating status

Cotton seed and synthetic aggregation pheromone of *T. castaneum* (4, 8-dimethyldacanal) (Insects Limited, Inc., Westfield, U.S.A.) (Suzuki 1980) were used as odour treatments to evaluate different aspects of the flight responses of beetles from each strain, and also the ability of the adults to locate those resources. Organic wheat flour (Kialla Pure Foods, Greenmount, Queensland) was also tested as an alternative resource in assessing the abilities of the beetles to fly and locate food.

As a measure of how the movement responses of the *T. castaneum* beetles differ across the test strains with respect to sex and mating status, 16 male and 16 female pupae of each strain were kept individually in plastic cups until their emergence as adults. One week after emergence, 8 each of these females and males were paired with a member of the opposite sex that had been marked with a dot of white nail polish. These pairs were held in individual plastic cups for 48 hours, so that they could mate. The unmarked adults were then separated out and their walking and flight activities were bioassayed. The remaining males and females were left unmated and were tested in the same way.

3.2.3. Wind tunnel assessments of flight

The flight responses of *T. castaneum* beetles from the different strains were measured in a rectangular, Perspex and push–pull airflow wind tunnel (120cm length × 60cm width × 50cm height) at 26 ˚C and 60 % r.h. The flight bioassays, with tests of the responses of the beetles

to food resources as well as a pheromone lure, were conducted between 1500h and 1800h (the usual flight time of these insects, Malekpour et al., 2016) with at least 80 beetles of each strain, during June, July and August 2015. The wind speed was 1.5 m/s, measured by a Testo 405-V1 anemometer (Instrument Choice, Adelaide, Australia) at the centre of the wind tunnel and a height of 5 cm. A thick cloth cone (8 cm tall) was placed inside a Petri dish (2 cm high × 10cm diameter) to provide a flight platform for the beetles, and this was positioned in the centre of the wind tunnel. The inner surface of the wall of the Petri dish was coated with Fluon® to prevent the beetles from walking up its sides.

The test material was placed on a filter paper, 50 cm upwind from the release point. A control test with a clean filter paper (no test material) was also included. Each individual adult was allowed to acclimate in the flight chamber for 5 min before the start of the experiment, by restraining it under a plastic cup in the Petri dish with the flight platform. The flight test with each individual beetle was run for 10 min and was recorded with a video camera (Panasonic, Tokyo, Japan) placed above the wind tunnel. If beetles did not fly within 10 minutes, they were recorded as not responding. EthoVision software (Noldus Information Technology, Wageningen, The Netherlands) was used to measure the flight speed of each individual beetle. Several other variables were also measured, including the number of beetles taking off in each experiment, the number of flying beetles orienting upwind towards the food resource, and the time to take-off. Sixteen observations were conducted daily with each sex of each strain included and with different food resources.

An additional test was run to determine the effect of mating status on the flight responses of the beetles. At least 8 individuals of each sex of each strain were tested in the wind tunnel in the way described above. Pheromone lure was placed upwind as it quickly stimulated flight

initiation (see results). Similar variables to those measured in the previous flight experiment were assessed to compare the flight responses of virgin beetles of each sex with those of mated ones.

3.2.4. Wind tunnel assessments of walking

The attraction of at least 80 walking beetles of each strain, towards the food resources and pheromone lure, was assayed in the wind tunnel (see above) during June, July and August 2015. Each test beetle was placed at the centre of the wind tunnel, 50cm upwind of the test odour stimulus. A video camera, positioned above the wind tunnel, recorded the walking tracks of each insect for 15 min. Daily assessments were run between 1000h and 1500h with different food resources and beetles of different sexes and strains. They were tested at this time because their tendency to fly is low during this period (Malekpour et al., 2016). Walking velocity (cm/s) was calculated with EthoVision tracking software. The number of beetles locating the food source or walking in any direction that ultimately took them away from the food source was noted. The time taken to locate the test resource was also measured. In addition, the responses of 8 mated beetles of each sex of each strain were recorded, while they were walking towards the pheromone source in the wind tunnel, in the same way as mentioned above, and these responses were compared with those from 8 unmated beetles of each sex of the same strain.

3.2.5. Statistical analyses

The flight and walking responses, with respect to odour stimuli and mating status, were compared between the field beetles and the beetles from the different laboratory strains by using a generalized linear model with a binomial distribution where the number of beetles was treated as a fixed factor. The effects of odour stimuli and mating status on walking and

flight responses were analysed by conducting a generalized linear model with a quasi-Poisson distribution where time was treated as a fixed factor. A linear model followed by ANOVA was fitted to test the walking speed of the field beetles and different laboratory strains where type of odour and mating status were treated as fixed factors. Walking speed data were analysed by a generalized linear model with a quasi-Poisson distribution where odour stimuli and mating status were treated as fixed factors. All the models were run in RStudio (R Development Core Team, 2013). The data from males and females were combined in those statistical tests in which sex had no significant difference (i.e. in most tests). Tukey's tests, the simultaneous test for general linear hypotheses, were conducted for post hoc multiple comparisons (Benjamini & Hochberg 1995).

3.3. Results

3.3.1. Flight response in relation to test resources

a. Number of beetles taking flight. The number of beetles of each strain that took flight is shown in Fig. 3.1. Beetles showed a significantly higher tendency for flight initiation in the presence of either a food resource or the pheromone lure, as compared with the blank control test (cotton seed vs control: GLM, $Z = 5.1$, $P < 0.001$, pheromone vs control: GLM, $Z = 6.01$, $P < 0.001$, wheat flour vs control: GLM, $Z = 2.9$, $P < 0.01$). Significantly, stronger responses were observed, within all strains, when the test odour stimulus derived from pheromones or cotton seeds (Fig. 3.1). Field beetles showed the highest responses in every treatment (and even in the controls) compared with the laboratory strains, including susceptible reference beetles (GLM, $Z = -2.8$, $P = 0.004$), resistant introgressed beetles (GLM, $Z = -5.1$, $P < 0.001$) and resistant reference beetles (GLM, $Z = -5.7$, $P < 0.001$). The overall flight activity of susceptible reference beetles was significantly higher than that of both resistant reference (Tukey's test, $Z = 3.3$, $P < 0.01$) and resistant introgressed beetles (Tukey's test, $Z = 2.6$, $P =$

0.03), but no significant difference was detected across the resistant strains in their flight responses (Tukey's test, Z = -0.7, P = 0.8) (Fig. 3.1). The flight responses of females were significantly higher than those of males (GLM, Z = -2.1, P = 0.03).

b. Time taken to initiate flight. Beetles of all strains initiated flight significantly sooner in the presence of odours (cotton seed vs control: GLM, T = -18.6, P < 0.001, pheromone vs control: GLM, T = -18.9, P < 0.001, wheat flour vs control: GLM, T = -17.8, P < 0.001) (Fig. 3.2 (top)). However, post hoc comparisons showed that the actual type of odour stimulus did not significantly affect the time taken (within strains) to start flight (Fig. 3.2 (top)). Field beetles did start to fly significantly sooner than any others after their release in the wind tunnel (field vs susceptible reference strains: GLM, T = 24.5, P < 0.001, field vs resistant introgressed strains: GLM, T = 31.9, P < 0.001, field vs resistant reference strains: GLM, T = 31.1, P < 0.001) (Fig. 3.2 (top)). No significant differences were detected between male and female responses in any of the treatments (GLM, T = -0.5, P = 0.5). So, the data representing male and female responses have been combined for analysis and representation in Fig. 3.2 (top).

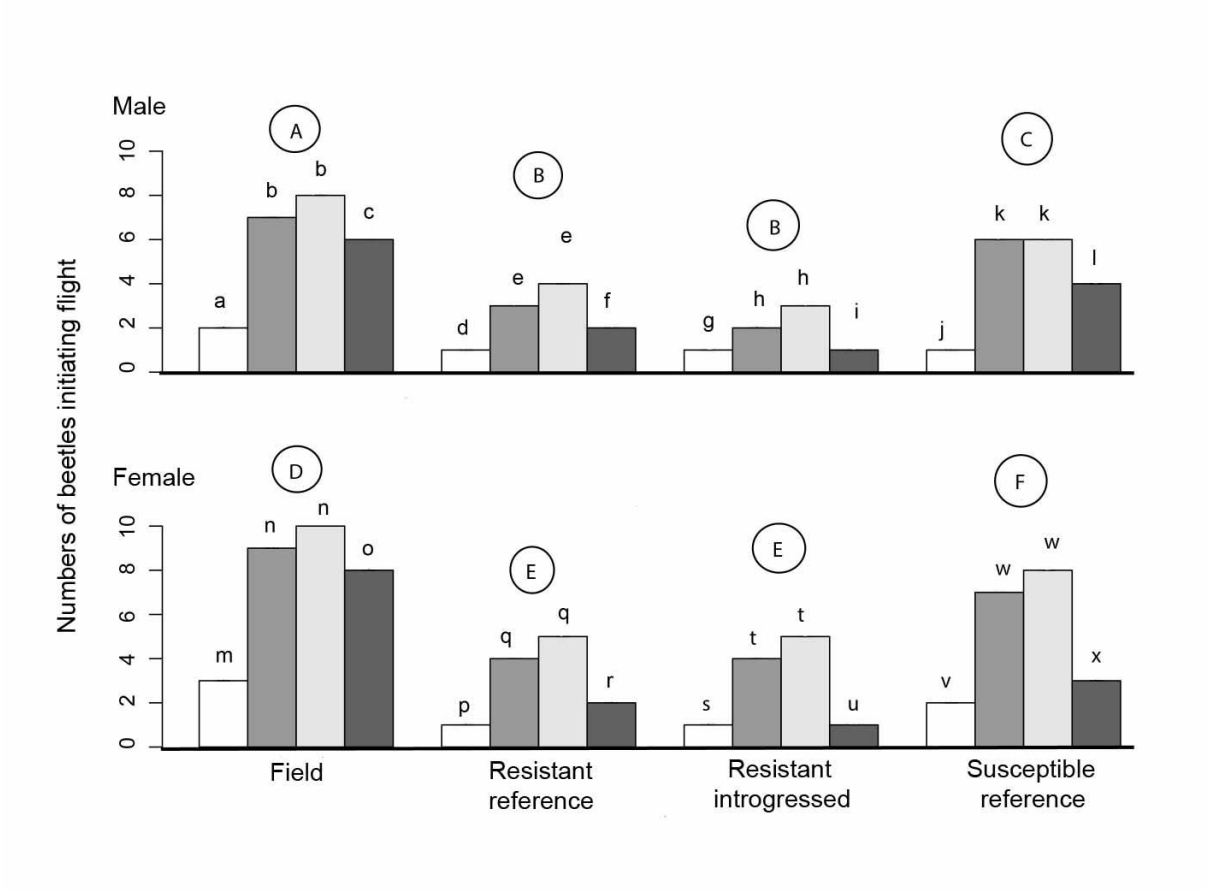

Fig. 3.1. Numbers of *Tribolium castaneum* adults of a field strain and three laboratory strains that flew in response to different odour stimuli, n = 10 for each sex of each strain. The colour of the histograms indicates each type of odour stimulus tested (white: no food, dark grey: cotton seed, light grey: pheromone, black: wheat flour). Results from post hoc pairwise comparisons across the strains are indicated with upper case letters above each set of bars, and the results of those tests across the odour stimuli within strains are indicated with the lower case letters immediately above each bar. Sets of histogram bars (within each diagram) that have the same letter are not significantly different from each other and the same is true of single bars within sets. The results of the statistical comparisons among the strains in response to each odour stimulus are given in the text. The sexes differed significantly from one another in their responses (see text).

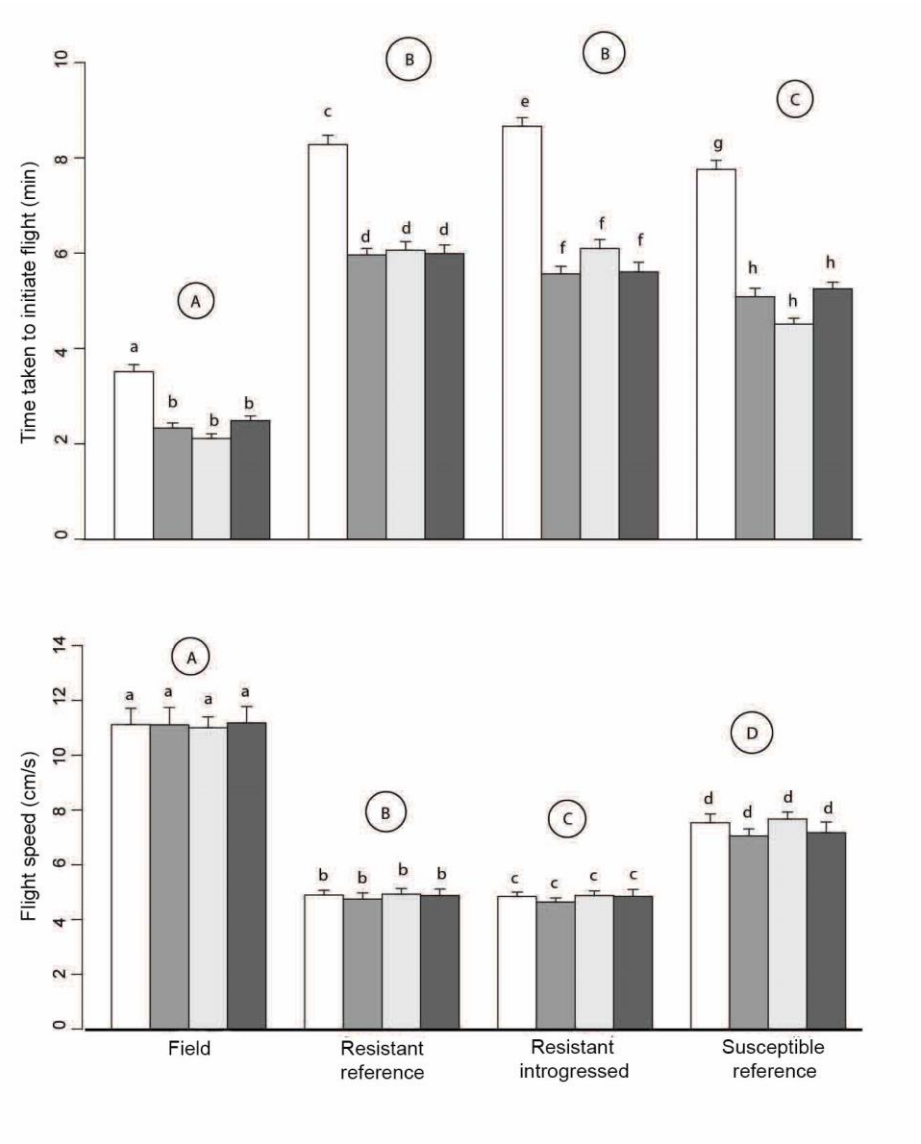

Fig. 3.2. Time taken (min) to initiate flight (top) and flight speed (cm/s) (bottom) of *Tribolium castaneum* adults of different strains that initiated flight in relation to different odour stimuli (n = 80 for each strain). The colours of the histogram bars represent the type of odour stimulus (white: no food, dark grey: cotton seed, light grey: pheromone, black: wheat flour). The upper case letters above each set of bars represent the results from Tukey's post hoc comparisons among strains, and the lower case letters above the bars themselves indicate the results from post hoc pairwise comparisons across odour stimuli within strains. The same letters indicate no significant differences across strains or across odour stimuli within strains, respectively.

c. Direction of flight. The presence of the test odour stimulus did not affect the direction of flight since all the beetles flew in the direction of the wind, as they did in the absence of odour stimulus (cotton seed vs control: GLM, $T = 0$, $P = 1$, pheromone vs control: GLM, $T = 0$, $P = 1$, wheat flour vs control: (GLM, $T = 1.2$, $P = 0.2$). Direction of flight was not significantly affected by sex (GLM, $T = -8.9$, $P = 0.3$), type of food odour (Appendix 3.1) on the presence of the phosphine resistance gene (Appendix 3.2).

d. Flight speed. Neither the type of odour stimulus (or absence of odour stimulus) (Appendix 3.3) nor beetle sex (GLM, $T = -0.2$, $P = 0.7$) affected significantly the flight speed within any of the strains. Field beetles flew significantly faster than the resistant introgressed beetles (GLM, $T = -25.3$, $P < 0.001$), resistant reference beetles (GLM, $T = -25.6$, $P < 0.001$) and susceptible reference beetles (GLM, $T = -15.2$, $P < 0.001$) (Fig. 3.2 (bottom)). Beetles carrying phosphine resistant genes flew significantly slower than susceptible reference ones (Appendix 3.4).

3.3.2. Flight response in relation to mating status

a. Number of beetles taking flight. The number of beetles that initiated flight was not significantly correlated with the mating status of adults in any of the strains (GLM, $Z = 0.4$, $P = 0.6$). Field beetles showed, significantly, the highest number of flight take-offs in every treatment compared with those of laboratory strains (field beetles vs susceptible reference strains: Tukey's test, $Z = -2.7$, $P = 0.03$, field beetles vs resistant introgressed strains: Tukey's test, $Z = -4.9$, $P = 0.001$, field beetles vs resistant reference strains: Tukey's test, $Z = -4.6$, $P = 0.001$). The numbers of beetles that initiated flight were significantly related to the presence of phosphine resistance genes (Fig. 3.3 (top)). Sex only marginally affected the number of beetles that started to fly (GLM, $Z = -1.7$, $P = 0.08$).

b. Time taken to initiate flight. Mating status was not significantly correlated with the time taken to flight initiation (GLM, T = -0.01, P = 0.9) nor was it different between male and female beetles (GLM, T = -0.6, P = 0.4). Field beetles initiated flight in a significantly shorter period compared with all laboratory stains (field beetles vs susceptible reference strains: Tukey's test, Z = 16.05, P < 0.001, field beetles vs resistant introgressed strains: Tukey's test, Z = 23.3, P < 0.001, field beetles vs resistant reference strains: Tukey's test, Z = 23.6, P < 0.001). However the time taken for resistant reference strain beetles to initiate flight was significantly longer than that taken by those from the susceptible reference strain (Fig. 3.3 (middle), Appendix 3.5).

c. Flight speed. The average speed of flight of each strain was not significantly affected by mating status (GLM, T = 0.01, P = 0.9) or by sex of beetles (GLM, T = -0.2, P = 0.8). Field beetles flew significantly faster than those from laboratory strains (field beetles vs susceptible strains: Tukey's test, Z = -8.3, P < 0.001, field beetles vs resistant introgressed strains: Tukey's test, Z = -13.9, P < 0.001, field beetles vs resistant reference strains: Tukey's test, Z = -13.9, P < 0.001). Flight speed was, rather, significantly affected by the presence of phosphine resistance genes, with genes being associated with slower flight (Fig. 3.3 (bottom)).

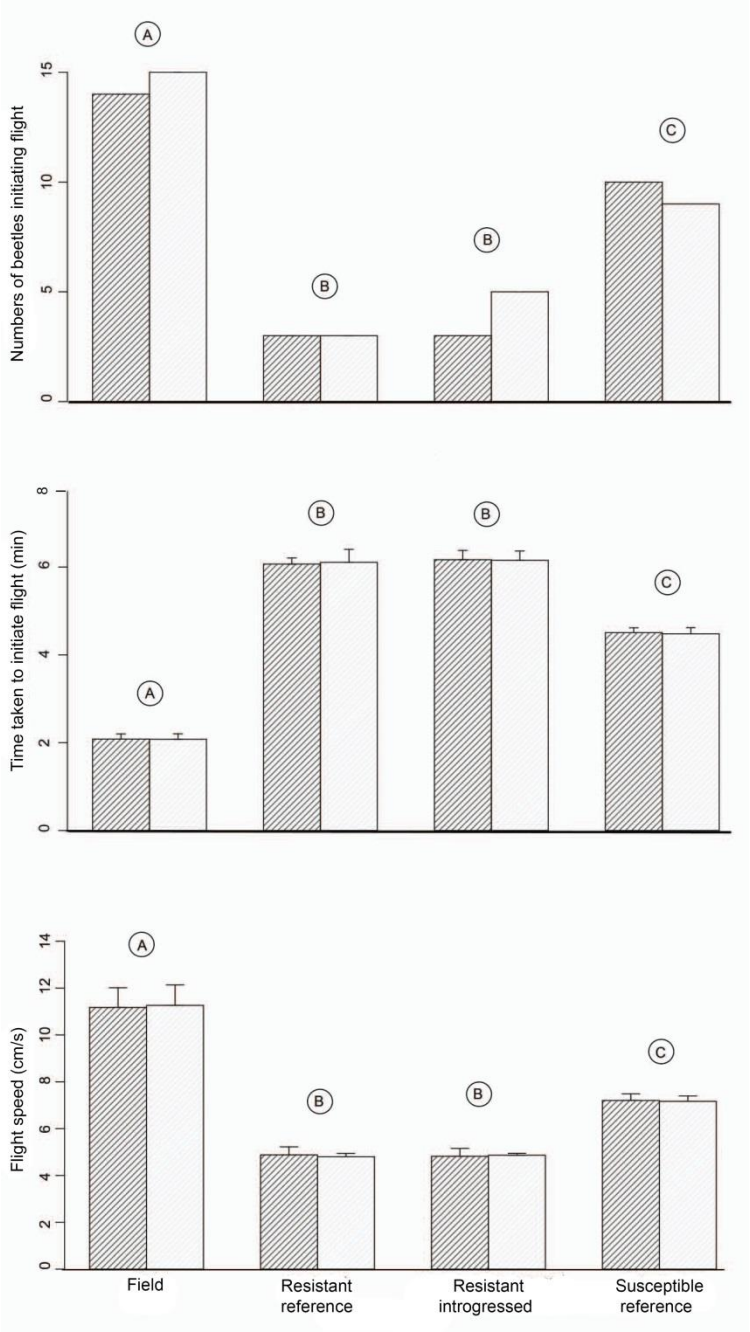

Fig. 3.3. Numbers (top) of *Tribolium castaneum* adults of a field and three laboratory strains that initiated flight in response to a pheromone lure are plotted in relation to their mating status. Dark and light cross hatched bars indicate mated and unmated beetles respectively [n = 8 of each sex of each mating status of each strain, but since sex only had a marginal effect on flight initiation, the related data are combined (n = 16 of each mating status of each strain)]. Also plotted are the time taken (min) to initiate flight (middle) and their flight speed (cm/s) (bottom). Indications of Tukey's post hoc pairwise comparisons are as for Fig. 3.1.

3.3.3. Walking response in relation to test lure

a. Number of beetles locating the lure. The number of beetles in the different treatments that located the lures is shown in Fig. 3.4 (top). The sexes have been combined because no significant differences were detected across male and female responses (GLM, $Z = 0$, $P = 1$). More beetles were significantly attracted by the cotton seed in the allocated time compared with wheat flour (GLM, $Z = -3.09$, $P = 0.001$), but no significant difference was evident between the attraction of beetles to the cotton seed and the pheromone lure (GLM, $Z = 1.1$, $P = 0.2$). The highest and the lowest numbers of beetles locating each lure came from the field adults and the beetles carrying phosphine resistance genes, respectively (Fig. 3.4 (top)).

b. Time taken to find the odour stimulus. No significant effect of sex on the time taken to locate the lure was detected (GLM, $T = -1.2$, $P = 0.2$). The beetles took significantly longer to find the wheat flour in the wind tunnel compared with the time to reach all the other lures (wheat flour vs cotton seed, Tukey's test, $Z = 23.9$, $P < 0.001$; wheat flour vs pheromone, Tukey's test, $Z = 22.5$, $P < 0.001$) (Fig. 3.4 (middle)). However, the time taken to locate the cotton seeds was not significantly different from the time taken to find the pheromone lure (Tukey's test, $Z = 1.4$, $P = 0.3$). Field beetles found the odour stimulus faster than did the laboratory strain beetles, including the susceptible reference beetles (GLM, $T = 11.9$, $P < 0.001$), the resistant introgressed strain beetles (GLM, $T = 23.8$, $P < 0.001$) and the resistant reference beetles (GLM, $T = 24.7$, $P < 0.001$). The results of a Tukey's test showed that beetles carrying phosphine resistance alleles took significantly longer to find the odour stimulus (resistant reference beetles vs susceptible reference beetles, Tukey's test, $Z = -13.02$, $P < 0.001$; resistant introgressed stain beetles vs susceptible reference beetles, Tukey's test, $Z = -12.09$, $P < 0.001$).

c. Walking speed. Beetles walked significantly faster in the presence of the odour stimulus compared to the control test (ANOVA, $F_{3, 312} = 9.4$). However, walking speed was found not to be significantly correlated with the type of food odour (pheromone vs cotton seed: Tukey's test, $T = -1.8$, $P = 0.2$; wheat flour vs cotton seed: Tukey's test, $T = -0.9$, $P = 0.7$; pheromone vs wheat flour: Tukey's test, $T = 0.9$, $P = 0.8$), as well as sex (ANOVA, $F_{1, 312} = 0.001$). Field beetles walked significantly faster than did the laboratory strain beetles (field beetles vs susceptible reference strains: Tukey's test, $T = -4.6$, $P < 0.001$, field beetles vs resistant introgressed strains: Tukey's test, $T = -16.3$, $P < 0.001$, field beetles vs resistant reference strains: Tukey's test, $T = -18.2$, $P < 0.001$). Walking speed was significantly different across susceptible reference and resistant reference strains (Fig. 3.4 (bottom)).

3.3.4. Walking response in relation to mating status

a. Number of beetles locating the lure. Mating status had a significant effect on the number of beetles responding to the pheromone lure (GLM, $Z = 2.8$, $P = 0.004$), with unmated beetles being significantly more strongly attracted to the pheromone than mated adults (Fig. 3.5a). However, the sex of the beetles did not influence their response to the pheromone lures (GLM, $Z = 0.8$, $P = 0.3$). In both treatments, mated and unmated, the field beetles had the highest number of individuals locating the food compared with the laboratory strains (field beetles vs susceptible reference strains: Tukey's test, $Z = -2.5$, $P = 0.04$, field beetles vs resistant introgressed strains: Tukey's test, $Z = -4.5$, $P < 0.001$, field beetles vs resistant reference strains: Tukey's test, $Z = -4.3$, $P < 0.001$). Among the laboratory strains, significantly more susceptible reference beetles located the lures, whether mated or unmated status (susceptible beetles vs introgressed strain: Tukey's test, $Z = 2.8$, $P = 0.02$, susceptible reference beetles vs resistant strain: Tukey's test, $Z = 2.5$, $P = 0.05$). The resistant reference

and introgressed resistant strains showed no significance in the number of beetles locating the lures (Tukey's test, $Z = 0.2$, $P = 0.9$).

b. Time taken to find pheromone lure. Although the unmated beetles of each strain were significantly quicker in locating the pheromone lure than were the mated beetles (GLM, $T = -9.4$, $P < 0.001$), no significant difference was detected in the time taken between males and females in finding the odour stimulus (GLM, $T = -0.2$, $P = 0.8$). Both mated and unmated field beetles located the pheromone lure in a significantly shorter time than their counterparts in the three laboratory strains (Fig. 3.5b). Among the laboratory strains, both unmated and mated beetles carrying the phosphine resistance genes took significantly longer to locate the pheromone source compared with their susceptible reference counterparts (Fig. 3.5b).

c. Walking speed. Walking speed was not significantly affected by mating status, nor by the sex of beetles (ANOVA, $F_{1, 122} = 1.3$ and ANOVA, $F_{1, 122} = 0.2$, respectively). Field beetles walked faster than the laboratory beetles (field beetles vs susceptible reference strains: Tukey's test, $T = -3.8$, $P < 0.001$, field beetles vs resistant introgressed strains: Tukey's test, $T = -11.6$, $P = 0.02$, field beetles vs resistant reference strains: Tukey's test, $T = -14.3$, $P < 0.001$) and among the laboratory beetles, susceptible reference beetles (whether mated or unmated) walked faster compared with resistant reference strain beetles (susceptible reference beetles vs introgressed strain: Tukey's test, $Z = 7.8$, $P < 0.001$, susceptible reference beetles vs resistant reference strain: Tukey's test, $Z = 10.5$, $P < 0.001$) (Fig. 3.5c).

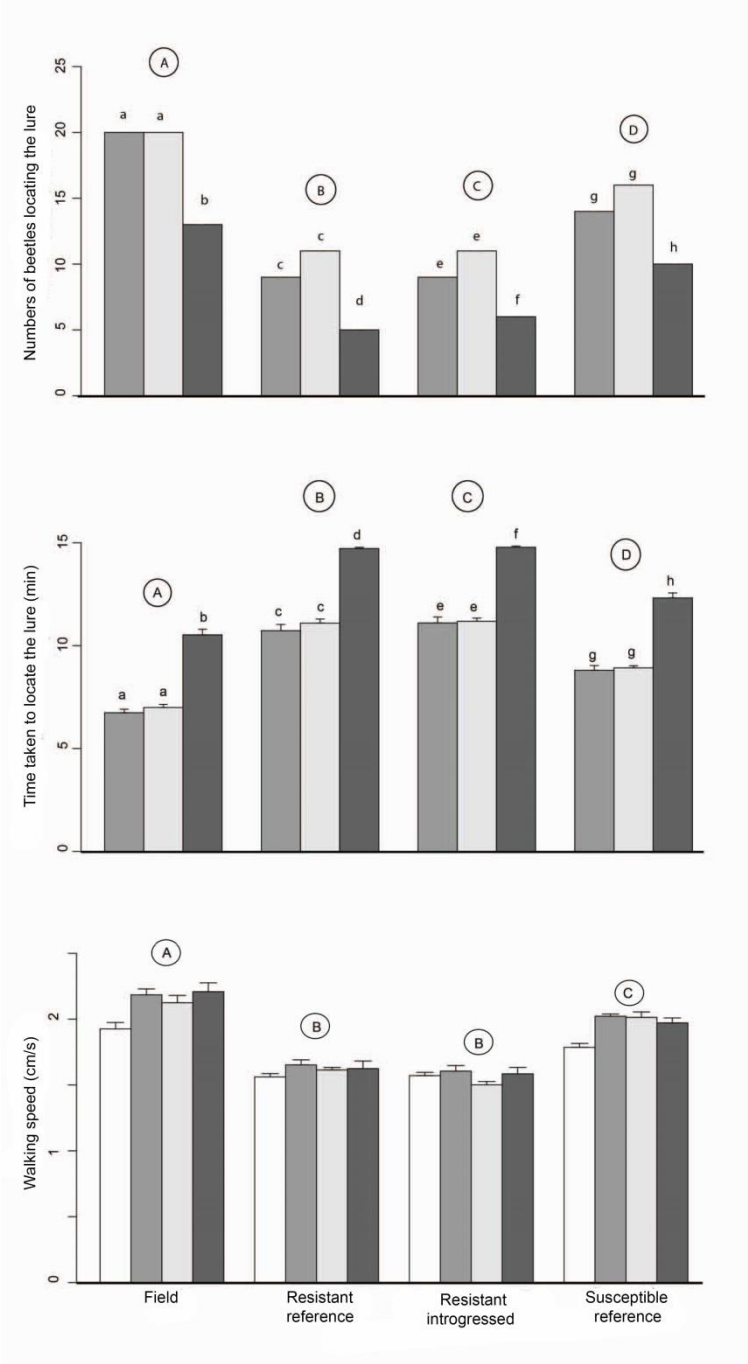

Fig. 3.4. Numbers (top), time taken (min) (middle) and walking speed (cm/s) (bottom) of *Tribolium castaneum* adults that located lures in relation to their phosphine resistance status (n = 80 for each strain). The colour of the histogram bars indicates the test resource involved (dark grey: pheromone, light grey: cotton seed, black: wheat flour). The upper case letters above the bars represent the results from Tukey's post hoc comparisons among strains, and the lower case letters above the bars indicate the results from post hoc pairwise comparisons across odour stimuli within strains, respectively. Sets of histogram bars with the same letters are not significantly different from each other.

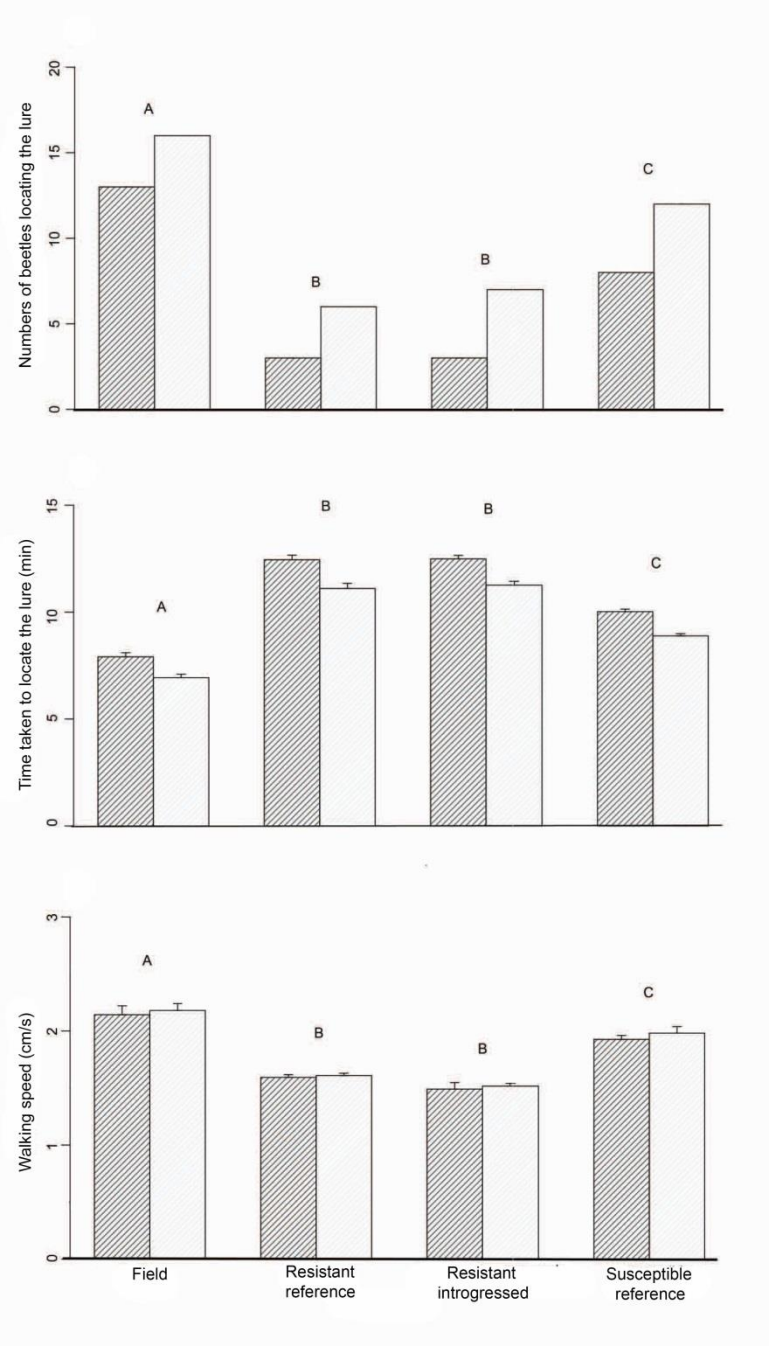

Fig. 3.5. Numbers (top), time taken (min) (middle) and walking speed (cm/s) (bottom) of adults of *Tribolium castaneum* locating lures in relation to their mating status [n = 8 of each sex of each mating status of each strain, but since sex had no significant effect on flight initiation of each strain, the related data are combined (n = 16 of each mating status of each strain)]. The shading within the histogram bars represents status of mating (dark cross hatched plot: mated beetles, light cross hatched plot: unmated beetles). Indications of Tukey's post hoc pairwise comparisons across strains are as for Fig. 3.1.

3.4. Discussion

Although the question of what makes organisms undertake dispersal activities is largely unanswered, interaction between the internal state of the individuals and environmental factors explain the basis of movement ecology, the fundamental trait of the organisms (Nathan et al. 2008). To know the impact of environmental factors on movement abilities, the attraction of susceptible reference and resistant reference *T. castaneum* beetles in relation to odours of various types was examined, and also their behaviour associated with their localization of those odours was studied. Differences observed in dispersal abilities between individuals are be likely to be linked with genetic control of this behavioural response (Edelsparre et al., 2014). The results of this study also have general implications for interpreting the flight behaviour of *T. castaneum* and for understanding the spatio-temporal dynamics of these insects in association with phosphine resistance genes.

The highest numbers of beetles initiated flight when odours were present, with an especially clear propensity for flight initiation when cotton seeds and the *T. castaneum* aggregation pheromone were upwind of them. These findings are consistent with the findings of Cox et al. (2007), who showed a significantly higher incidence of flight in *T. castaneum* as well as other pests of stored products when groups of beetles released in the cylindrical flight chamber in the presence of food odours. Perez-Mendoza et al. (2011b) tested the effect of the presence or absence of food on individual flight initiation (as I did) within flight chambers constructed from Petri dish. In contrast to these results, they reported that the presence or absence of food in these small flight chambers did not affect the rate of flight initiation. They tested the beetles in small confined Petri dish with no airflow, while I tested individual beetles within a wind tunnel. The reason for the difference in results could be because wind-borne odours induced flight initiation of the beetles in the wind tunnel. This research

indicated that although food odours did not induce upwind flight, beetles initiated to fly much sooner in the presence of food odours regardless of the type of food resource (Fig. 3.2 (top)).

No mating status differences were detected in flight initiation of all strains tested. Perez-Mendoza et al. (2011a) also found that mating status did not affect flight initiation of *T. castaneum* and suggested their flight activity is a mechanism to disperse, but is not related to the proposed oogenesis-flight syndrome of migratory insects (Johnson 1963). Females showed the greatest propensity to take flight (on average about 1.3 times than males) (Fig. 3.1). This finding is consistent with the results of a laboratory study conducted by Levinson & Mori (1983) and the field trapping results of Ridley et al. (2011), who captured >50% females in pheromone trap. However, no differences were reported in flight tendency between females and males in the laboratory experiments by Perez-Mendoza et al. (2011a, b). Similar to the result of previous work, significantly more susceptible beetles initiated flight compared with beetles known to carry phosphine resistant alleles (Malekpour et al., 2016). According to the results of this study on walking, both sexes of *T. castaneum* were attracted more strongly to the pheromone lure and cotton seeds. Therefore shorter time was taken to find these two resources compared with wheat flour. These results are consistent with the findings from short-range resource localization studies conducted within laboratory and glasshouse conditions (Ahmad et al. 2013).

Unmated beetles showed a faster and much stronger attraction towards resources compared with the mated beetles. Higher number of susceptible reference beetles in a shorter period of time located the resources than those of beetles carrying phosphine resistant genes. Food location did not seem to be associated with sex. Beetles walked faster in the presence of food resource. However, flight speed and walking velocity of all strains were not affected by the

resource type, sex and mating status. Higher speed of flight and walking was observed among susceptible reference beetles compared with the phosphine resistant reference beetles. Furthermore, lower ability in activity both flight and walking of the laboratory beetles compared with field beetles may result from the short-term consequences of selection forces under laboratory conditions. Because beetles cultured in the laboratory have continued access to high quality food resources without any need to disperse and locate resources.

The results have implications for understanding the spatial distribution of phosphine resistance in the field. In particular, the lower movement ability both by walking and flight of the phosphine-resistant reference beetles (Malekpour et al., 2016) leads to the undertaking migratory flight at a relatively lower frequency than susceptible reference beetles and being less efficient (with respect to time) in locating to the resources in the field. Although the food resource that was not most attractive to phosphine resistant reference beetles was the same as for susceptible reference ones, the resistant reference beetles took much longer time (1.6 times) to find the food than did the susceptible reference ones, which is attributable to the pleiotropic effect of the phosphine resistant genes on the movement abilities of these insects. It is likely that the life of resistance beetles strongly will be affected in the field, because they don't locate resource effectively and also fly less. Moreover, this study indicates that mating status has a similar effect on both walking and flight of resistant reference strain beetles compared with that of the susceptible reference ones. Unmated beetles of the resistant reference strain walked more than mated beetles of this strain, as was for susceptible reference ones. This greater response might result from higher motivation of unmated beetles to find mates. Flight of both susceptible reference and resistant reference strains was not associated with mating status. *T. castaneum* uses flight as a dispersal mechanism at any time of their life span and not be linked to the mating status. Therefore both intrinsic and

environmental factors have similar effect on flight propensity and resource location abilities of resistance reference and susceptible reference strains. Variation in all these features means that each behaviour occurs with a certain probability rather than with any certainty, even though there is a considerable genetic influence on all of these.

Chapter 4

The effects of sublethal phosphine exposure on the respiration and dispersal propensity of susceptible and resistant strains of *Tribolium castaneum*

4.1. Introduction

The rust red flour beetle, *Tribolium castaneum* (Herbst), is a serious pest that has shared a long association with stored food grains and a wide range of processed commodities in the tropics and subtropics (Bell, 2000). Phosphine gas has been employed as the method of choice for the chemical control of grain pests for over half a century (Chaudhry, 2000). The action of phosphine against insects differs from that of other fumigants in that longer exposures to phosphine, at relatively low dosages, are more effective than shorter exposures at higher concentrations of the gas (Chaudhry and Price, 1990b, Hole et al., 1976). Indeed, higher concentrations of phosphine alone induce narcosis in the insects, which incidentally protects them from the toxic effects of the phosphine gas (Hole et al., 1976). Thus, extended exposure periods can be considered the main strategy to achieve maximum effectiveness of phosphine fumigation against pests (Benhalima et al., 2004). Research on several pest species shows that any change in exposure time, dose and temperature (whether independently or in combination) leads to a change in effective control of insect infestations (Nayak and Collins, 2008). For example, the toxicity of phosphine is reduced by a decrease in temperature, because that reduces the metabolic rate of the target insects, and that results in lower uptake rates of the fumigant (Chaudhry et al., 2004).

The usefulness of fumigation with phosphine gas became seriously threatened with the detection of genetic resistance to the action of phosphine in the 1970's for the first time, and then the more regular acquisition of resistance genes in other parts of the world in several grain pests (Ahmedani et al., 2007). Resistance to phosphine has developed in response to the selection pressures generated by repeated ineffective fumigations, usually in situations where the gas leaks rapidly from the storage facilities contains bulk of treated grains. Poorly sealed structures in warehouses are common in several parts of the world (Bell, 2000; Mills, 1983).

Sublethal phosphine exposure decreases the efficacy of phosphine use, and because this allows individuals to survive, increased application of the fumigant follows (Chaudhry, 2000). Such repeated application of sublethal doses allows individuals with resistance alleles to survive, and so the build-up of resistant populations follows (Chaudhry, 2000; Mills, 1983). Sublethal phosphine exposure also affects insect behaviour, which alters the tolerance of the individuals and influences control efficacy (Bond and Upitis, 1973). *Rhyzopertha dominica* adults exposed to sublethal doses of phosphine showed a temporary suppression in reproduction for one to two weeks (Ridley et al., 2012). Exposure to sublethal doses of phosphine also resulted in a distinct delay in the egg hatching rates of the psocid *Liposcelis bostrychophila* (Ho and Winks, 1995; Nayak et al., 2003).

Mitochondria, the cellular sites of aerobic respiration, are recognised as the site of phosphine action (Nath et al., 2011, Valmas et al., 2008). Phosphine toxicity is associated with the disruption of chemical reactions involving oxygen and results in inhibition of respiration (Chefurka et al., 1976, Price and Dance, 1983). Understanding of how sublethal exposure to phosphine affects insect respiration is important for predicting its effects on energy demanding activities, including movement. Treatment with a sublethal dose of phosphine can lower the rate of oxygen consumption (Hobbs and Bond, 1989) and reduces insect mobility (Pimentel et al., 2012). The problem with these altered behaviours is that fumigation may even seem to have been successful when insect survival has, instead, not decreased much at all (Nayak et al., 2003). This study is, therefore, focused on understanding how sublethal phosphine exposure affects the metabolic rate and movement abilities of *T. castaneum* beetles.

Four populations of *T. castaneum* were used in the present study. These populations differed from one another in their phosphine resistance status, and were fumigated with a sublethal concentration of phosphine. The aim of the present study was to evaluate the respiration and movement responses of surviving insects after recovery from a sublethal dose of phosphine. Also, any differences across the four populations would contribute to understanding the fitness costs of phosphine resistance, because of their different resistance levels. An understanding of these effects is fundamental to improving the efficacy of phosphine use in stored-product fumigation and for the development of more effective integrated pest management programmes against stored-product pests and in managing the resistance of these insects to phosphine.

4.2. Materials and methods

4.2.1. Insect populations

Sublethal treatments were conducted with field beetles and three laboratory populations of *T. castaneum*, including the susceptible reference strain, resistant introgressed strain and resistant reference strain, described in an earlier publication (Malekpour et al., 2016). The field beetles were established in the laboratory from a minimum of 100 adults collected from wheat silos near Dalby (27° 11′ 0″ S and 151° 16′ 0″ E), west of Brisbane, Queensland, Australia in mid-2015. These beetles were cultured on wholemeal wheat flour, supplemented with 5% torula yeast, for five months and maintained at 26°C and 46% RH and 12:12 L: D photoperiod. Female pupae were separated from the culture under a dissecting microscope, by examining the size of their external papillae (Halstead, 1963), and placed alone within the wells of an ELISA plate with a sprinkling of wheat flour to allow them to complete their development. Newly-emerged females (14 days) were used in the experiment.

The laboratory strain cultures were established from cultures held by the Postharvest Grain Protection Team (Department of Agriculture and Fisheries, Brisbane, Queensland, Australia). The susceptible reference strain "QTC4", collected from a storage facility in Brisbane, has been reared under laboratory conditions in the absence of selective pressures from insecticides since 1965 (Jagadeesan et al., 2012). The resistant reference strain "QTC931" was established with phosphine resistant individuals collected from southern Queensland in 2000 (Jagadeesan et al., 2012). The resistant introgressed strain "ISOTC24" was developed from the resistant reference and susceptible reference strains by a process of crossing and then selection with phosphine exposure (Daglish, unpublished data, Malekpour et al., 2016). These beetles were reared under similar conditions to those described above for the field beetles. Each individual beetle was tested for flight propensity, lure finding behaviour and metabolic rate, both before and after fumigation with phosphine, all of which is described below.

4.2.2. Sublethal phosphine exposure (fumigation procedure)

4.2.2.1. Pre-fumigation procedure

a. Phosphine gas generation

Phosphine (ca. 86% pure) was generated in an apparatus in a fume cupboard. The top of a collection tube was submerged completely in a glass container filled with 5% sulphuric acid solution to remove all air from inside the collection tube. A tablet of aluminium phosphide, wrapped in filter paper and then gauze, was dropped into the solution beneath an upturned glass funnel. The collection tube was positioned over the stem of the glass funnel, which pointed upward. Generation of phosphine gas commenced immediately and could be observed as gas bubbles forming within the solution. Phosphine gas left the upturned funnel through the vertical stem and displaced the acidified water in the collection tube. After 24

hours, the phosphine gas was ready for use and could be drawn up using a syringe for use elsewhere.

b. Measuring phosphine concentration

The phosphine concentration was verified before fumigation of the beetles with gas chromatography (GC) using a thermal conductivity detector (TCD) (Clarus 500, PerkinElmer, Shelton, U.S.A.). A 30 µl sample from the collection tube was injected into the GC with a gas-tight syringe. The concentration of phosphine was estimated by the mean of five to eight samples taken directly from the phosphine source.

c. Determining the volume of desiccators

Calculating the desiccator volume was necessary for calculating the correct volume of phosphine gas to be injected into each desiccator. Each fully assembled desiccator was therefore filled with water before the tests were conducted. The weight of water (g) in each desiccator gave its volume (ml).

d. Measuring the volume of phosphine gas to be injected

A determination of the correct phosphine volume for injection into the fumigation desiccator was based on the concentration of phosphine measured using GC, the desiccator volume, and the required dose of phosphine for each of the strains. The following equation was used:

$d_1\,(\mu l) = x_1\,(mg/l) \times v_1\,(l) / x_2\,(mg/l) \times 1000 \times 1000$

Where d_1 (µl) = volume of phosphine gas to inject, x_1 (mg/l) = required dose of phosphine in desiccator, v_1 (l) = volume of desiccator and x_2 (mg/l) = concentration of phosphine source.

4.2.2.2. Fumigation

Fumigation of adults (FAO, 1975) took place at 25°C and over an exposure time of 20 h. Each female beetle, whose movement behaviour and metabolic rate had been measured (see below), was confined in a plastic cup sealed with a lid with numerous tiny holes. All of the plastic cups with beetles that had to be fumigated with a similar concentration of phosphine were placed inside a gas-tight desiccator (the fumigation chamber) fitted with a rubber septum in the lid. A known quantity of phosphine from the source was injected, with a gas-tight syringe, through the rubber septum to produce a dose to approximate the LC_{10} for each strain. Because the tolerance of the susceptible reference and resistant reference strains differs, different quantities of phosphine gas were injected into each desiccator to produce the desired concentration for each. The LC_{10} values were 0.004, 1.000 and 0.800 mg/l for the susceptible reference, resistant reference and resistant introgressed strains, respectively. A control test with 50 beetles of each strain (not exposed to phosphine) was set up to estimate the mortality rate expected under normal conditions. After the required exposure period (20 h), the lids of the desiccators were removed and the plastic cups extracted from the desiccators under a fume hood cupboard. The beetles were fed with a small quantity of culture medium added to each plastic cup and were maintained in this way for 14 days (25°C and 46 % RH), when mortality was assessed and the living beetles were again assessed for flight, lure finding behaviour and metabolic rate.

The initial fumigation of the resistant introgressed strain beetles killed most of them. This unanticipated high mortality rate could have resulted from the physiological condition of these beetles, or inadvertent misjudgement of dose, or a problem with the fumigation process. The fumigation procedure was therefore conducted a second time with a larger number of beetles of this strain. If the problem was with the physiological condition of these beetles,

fumigation a larger number of beetles could help get enough number of survivors after fumigation. Simultaneously, beetles of the resistant reference strain were also fumigated to ensure the fumigation process itself was not the problem. If the beetles of the resistant reference strain died after fumigation, it would indicate that fumigation process needed attention. At the end of the second week after exposure, the number of survivors was counted and the data on the survivors of the second fumigation were used in the subsequent test and the statistical analyses involving this strain.

4.2.3. Pre- and post-sublethal phosphine exposure assays

a. Metabolic rate

The rate of CO_2 production by individual beetles was used as a proxy for their metabolic rate (Lighton, 2008) and was measured for each of the 15 beetles of each strain tested above, at 25±1°C, using flow-through respirometry. Room air was passed sequentially through columns containing soda lime (Ajax Finechem Pty Ltd, Taren Point, NSW, Australia) to remove CO_2 and Drierite (Sigma-Aldrich Co., St. Louis, MO, U.S.A.) to remove water vapour. The flow rate through the chamber was regulated at 30 ml/min by a pump (TR-SS3, Sable Systems International, Las Vegas, NV, U.S.A.) and a mass flow controller (GFC17, Aalborg Instruments & Controls Inc., Orangeburg, NY, U.S.A.). The dry and CO_2-free air was then passed through a metabolic chamber housing an individual beetle before being passed through a single-channel LI-820 CO_2 analyser (Li-COR Inc., NE, U.S.A.). The LI-820 detected the CO_2 concentration in the excurrent air (produced by the individual beetle's respiration) and was interfaced with Li-COR software to plot and record the CO_2 trace with a 1 ppm resolution and 1 Hz recording frequency. The metabolic chamber was a short glass cylinder of 2 ml in volume, made airtight using drilled rubber bungs on either end and connected to the incurrent and excurrent airline tubing.

The metabolic chamber was held within a locomotion activity monitor (LAM10H; TriKinetics Inc., Waltham, MA, U.S.A.) that had nine infrared LED emitter-detector pairs. Each movement by the beetle that interrupted the infrared beam was detected and counted by the activity monitor. This allowed synchronous measurement of spontaneous activity with metabolic rate, as *T. castaneum* adults are relatively active even under typical resting metabolic rate conditions (Arnold et al., 2016). Therefore, this trait was considered to be measuring routine metabolic rate (routine MR); the lowest metabolic rate of a post-absorptive, thermoneutral, and undisturbed adult ectotherm, but allowing for some level of activity (Mathot and Dingemanse, 2015). Once an adult beetle was placed in the metabolic chamber, the CO_2 produced by each individual beetle was measured for 30 min at 25°C in darkness, and then a 20 min subsample of the 30 min routine MR was taken. Each individual beetle was weighed to 0.01mg on a microbalance (XS3DU; Mettler-Toledo, Columbus, OH, U.S.A.) directly following its routine MR measurement.

b. Flight propensity

A rectangular perspex wind tunnel (120 cm length × 60 cm width × 50 cm height) was used to measure the flight propensity of different strains of *T. castaneum* during December 2015. The flight bioassays were run with 15 females, of each strain, between 1500h and 1900h, the time at which they fly most readily (Malekpour et al., 2016, Perez-Mendoza et al., 2011a). A flight platform, comprising a thick cloth cone (8 cm height), was placed in the centre of the wind tunnel, inside a Petri dish (2 cm high × 10 cm diameter) to prevent the beetles from walking in the wind tunnel. The inner surface of the wall of the Petri dish was coated with Fluon® to prevent the beetles from walking up its sides. Pheromone lures were placed upwind, 50 cm from the release point, to stimulate flight, as established by Malekpour et al.

(2016). To carry the odours towards the beetles, wind was pushed through the tunnel by a variable speed fan set at 1.5 m/s. A Testo 405-V1 anemometer (Instrument Choice, Adelaide, Australia) was used to check the speed of wind at the centre of the wind tunnel and at a height of 5 cm. Each individual adult was released alone in the flight chamber 5 min prior to the start of the experiment, to acclimate. Sixteen observations were conducted daily (four beetles of each strain), and a test was ended if the beetle had not flown within 10 min and this was recorded as the beetle not having flown.

c. Lure locating behaviour

The walking bioassay was conducted with the same 15 females of each strain whose flight propensity was examined, and this was done the day after the flight test was conducted. Each beetle was introduced individually in the centre of the wind tunnel described above. Assessments were run between 1000h and 1500h, the time at which very little flight occurs (Malekpour et al., 2016). New pheromone lures (as above) were placed upwind, 50 cm from the release point of the beetles. Beetles were recorded as having located the lure in 10 min, or not.

4.2.4. Phosphine resistant test for field-cultured beetles

Two discriminating dosages were used to determine the phosphine resistance status of the field-collected beetles that cultured for only one or two generations. Beetles were divided into four groups of 50 individuals. Two groups were exposed to a high dose of phosphine (0.25 mg/l) and the other two groups were fumigated with a low dose of phosphine (0.04 mg/l) for 20 h in each case. The lower dose diagnosed the resistance or susceptibility of the beetles, and the higher dose determined the level of resistance (Table 4.1). The fumigation procedure

was as described above. Mortality was assayed after the recovery period, which was again 14 days.

4.2.5. Statistical analysis

All data analyses were conducted with the R 2.15.1 statistical software package (R Development Core Team, 2013). Routine MR data were analysed using a generalized linear model (GLM) with a Poisson distribution, and with fumigation, phosphine resistance status, mass and activity being treated as fixed factors. For flight and lure finding data, a generalized linear effects model (GLM) with a binomial distribution was fitted to test the effect of mass, fumigation and phosphine resistance status on them. Tukey's post hoc pairwise comparisons were also performed using the *glht* function of the multicomp package with adjusted *p* values to examine if specific pairwise comparisons between treatments were significantly different. Differences between numbers or percentages of beetles that flew before and after fumigation, and also numbers or percentages of beetles that located the food before and after fumigation, were compared with a Pearson's chi-square test.

Table 4.1. Interpretation of test fumigation results, designed to determine the phosphine resistant status of field collected *Tribolium castaneum* beetles.

Low dose	High dose	Classification
No survivors	No survivors	Susceptible
Survivors	No survivors	Weak resistant
Survivors	Survivors	Strong resistant

4.3. Results

4.3.1. Mortality assessment

Among all strains, fumigation was most effective against resistant introgressed strain beetles, with 20% mortality followed by resistant reference strain beetles, with 15% mortality. The efficacy of fumigation was least for field beetles (5% mortality rate) and susceptible reference strain beetles (10% mortality rate).

4.3.2. Metabolic rate

Figure 4.1 shows the CO_2 production of female individuals before exposure to phosphine and after exposure to phosphine. Before fumigation, the routine MR of the field beetles was significantly lower from that of all three strains of laboratory beetles, namely the resistant reference strain (GLM, $Z = 2.8$, $P = 0.004$), resistant introgressed strain (GLM, $Z = 2.6$, $P = 0.008$) and susceptible reference strain (GLM, $Z = 4.8$, $P < 0.001$) (Fig. 4.1A). The susceptible reference strain beetles had the highest routine MR among all tested strains (susceptible strain vs field beetles: Tukey's test, $Z = 4.8$, $P < 0.001$, susceptible reference strain vs resistant reference strain: Tukey's test, $Z = 3.3$, $P = 0.003$, susceptible reference strain vs resistant introgressed strain: Tukey's test, $Z = 4.1$, $P < 0.001$). No significant difference in routine MR was detected between resistant reference strain and resistant introgressed strain beetles (Tukey's test, $Z = -0.4$, $P = 0.9$).

A significant decline in routine MR was detected in the beetles of all strains after fumigation (GLM, $Z = 3.4$, $P < 0.001$) (Fig. 4.1B). Body mass (Fig. 4.2) was significantly related to routine MR before fumigation (GLM, $Z = 1.9$, $P = 0.04$), however, no such correlation was detected after fumigation (GLM, $Z = 0.6$, $P = 0.4$). Spontaneous activity, which was measured synchronously with routine MR, was not significantly related to the routine MR of

the beetles, neither before fumigation (GLM, Z = -1.1, P = 0.2) nor after fumigation (GLM, Z = -0.07, P = 0.9).

4.3.3. Flight propensity

The number of female adults of each strain that initiated flight before and after fumigation is shown in Fig. 4.3. Before fumigation, the resistant reference strain beetles took off for flight significantly less than the field beetles ($\chi 2$ = 16.2, df = 1, P < 0.001), and also less than did the susceptible reference strain beetles and resistant introgressed ones, but not significantly ($\chi 2$ = 1.53, df = 1, P = 0.2, $\chi 2$ = 0.2, df = 1, P = 0.6 respectively) (Fig. 4.3A). The exposure of the beetles to sublethal doses of phosphine decreased the flight initiation frequency significantly, in all strains, by a factor of 5-30%, depending on strain (GLM, Z = 2.1, P = 0.02) (Table 4.2). Fumigation significantly affected the rate of flight initiation of field beetles ($\chi 2$ = 21.12, df = 1, P < 0.001) and resistant introgressed beetles ($\chi 2$ = 4.5, df = 1, P = 0.03) (Table 4.2).

The flight initiation pattern, across strains, of adults after fumigation was similar to that of beetles before fumigation (Fig. 4.3). Body mass had no significant effect on the number of beetles initiating flight before fumigation (GLM, Z = -0.04, P = 0.9) or after (GLM, Z = -0.7, P = 0.4). The routine MR recorded for each beetle also had no significant effect on its propensity for flight, whether before fumigation (GLM, Z = 0.4, P = 0.6) or after (GLM, Z = -1.02, P = 0.3).

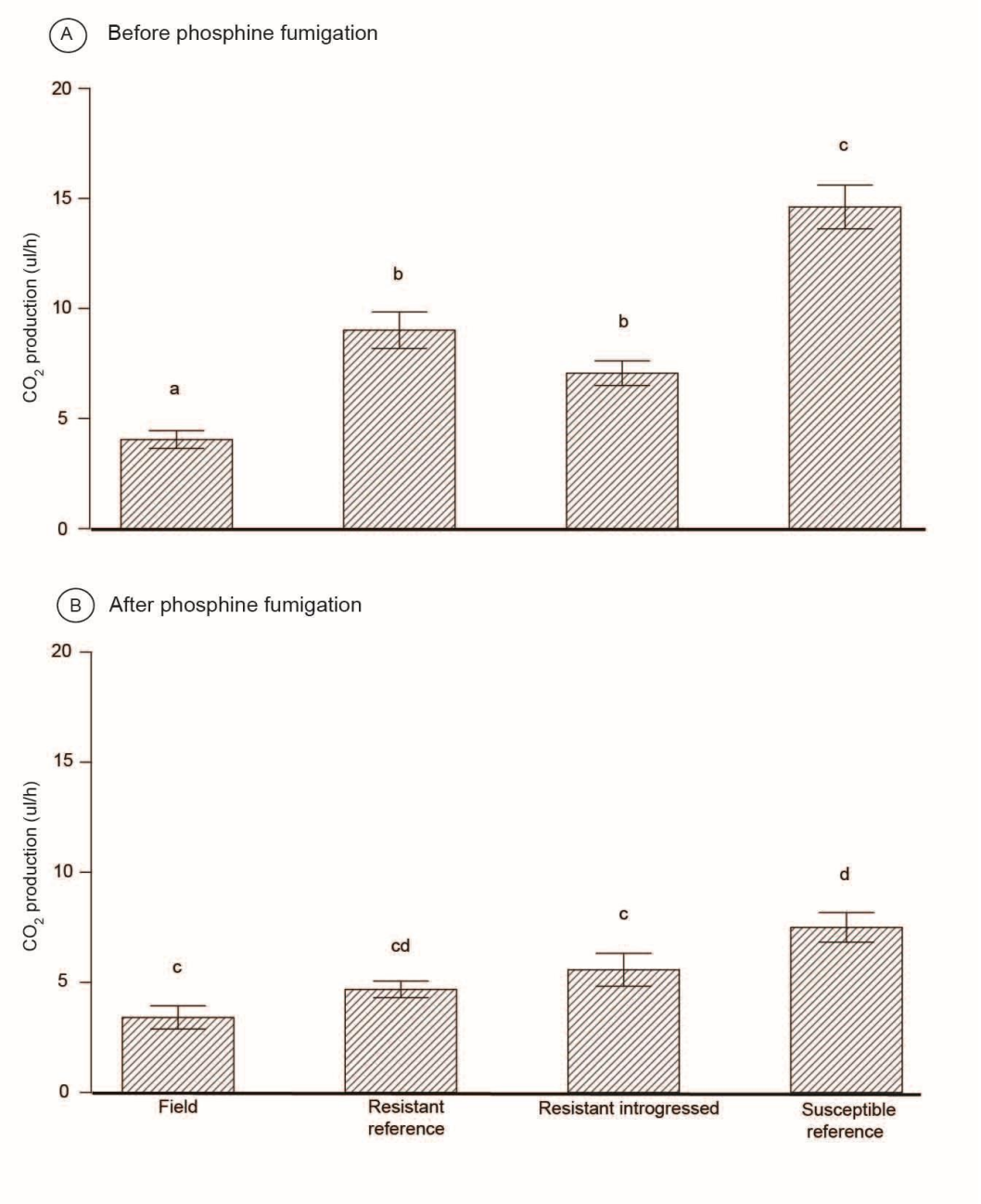

Fig. 4.1. Routine metabolic rate (µl CO_2 h^{-1}) of individual *Tribolium castaneum* adult females from a field strain and three laboratory strains (resistant reference, resistant introgressed and susceptible reference strains) before fumigation (A) and after fumigation (B), n = 15 for each strain. Results from post hoc pairwise comparisons across the strains are indicated with lower case letters above each bar. Within each of these comparisons, bars with the same letter are not significantly different from each other.

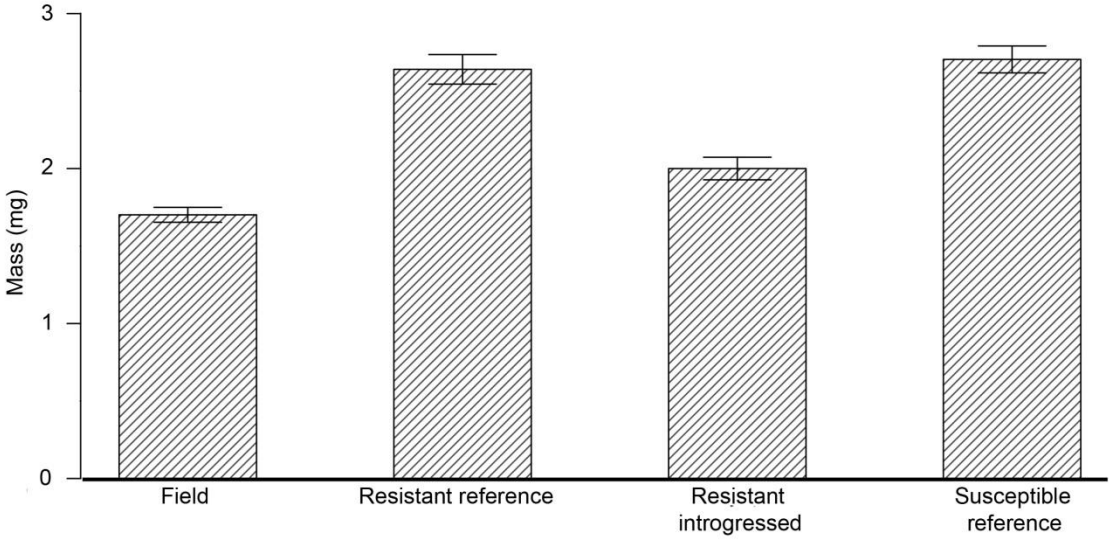

Fig. 4.2. Mean mass (mg) of individual *Tribolium castaneum* adult females from a field strain and three laboratory strains (resistant reference, resistant introgressed and susceptible reference) tested for metabolic rate, n = 15 for each strain.

Table 4.2. Percentage of beetles that initiated flight before and after fumigation, n = 15 in each treatment. Significant statistical differences in the percentage of beetles that initiated flight before and after fumigation have been indicated by different superscripts (Chi-square test on raw data).

	Percentage of beetles initiating flight	
Strains	Before fumigation	After fumigation
Field strain	93.5 [a]	66.5 [b]
Resistant reference strain	13.5 [a]	6.5 [a]
Resistant introgressed strain	26.5 [a]	13.5 [b]
Susceptible reference strain	40.0 [a]	33.5 [a]

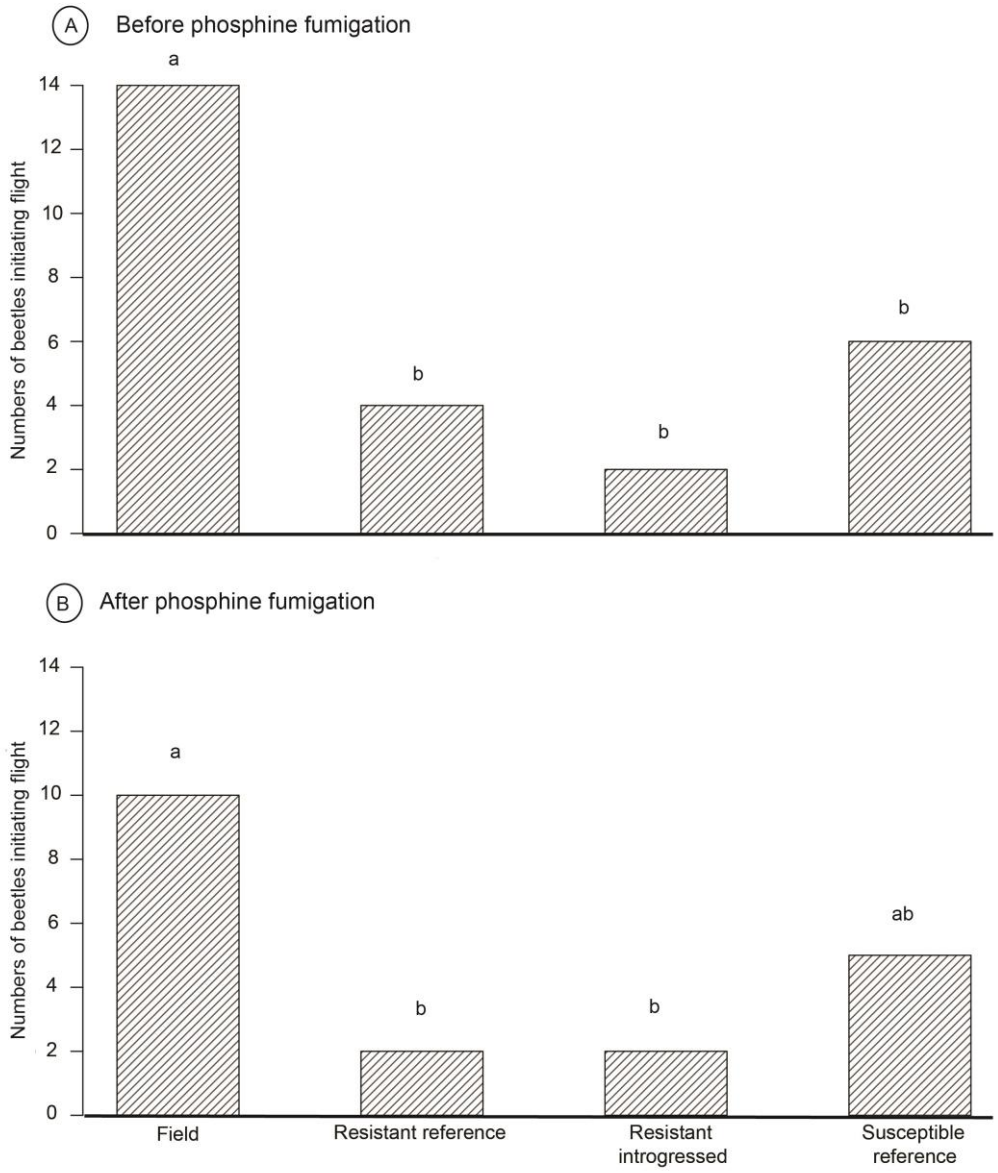

Fig. 4.3. Numbers of *Tribolium castaneum* female adults of a field strain and three laboratory strains that flew in response to a *T. castaneum* aggregation pheromone lure before fumigation (A) and after fumigation (B), n = 15 for each strain. Results from post hoc pairwise comparisons across the strains are indicated with lower case letters above each bar. Within each of these comparisons, bars that have the same letter are not significantly different from each other.

4.3.4. Lure locating behaviour

The number of beetles that were successful in locating the pheromone lure before and after fumigation is shown in Fig. 4.4. Prior to fumigation, field beetles were significantly more successful beetles at finding the lure relative to beetles of the phosphine resistant reference strain ($\chi 2 = 8.6$, df = 1, $P = 0.003$), resistant introgressed strain ($\chi 2 = 5.1$, df = 1, $P = 0.02$) and somewhat more than the susceptible reference strain, although the latter was not significantly different ($\chi 2 = 0.2$, df = 1, $P = 0.6$) (Fig. 4.4A). Among the laboratory strains, the susceptible reference strain beetles were the most successful in lure locating, however the difference was not significant (susceptible reference strain vs resistant reference strain: $\chi 2 = 4.8$, df = 1, $P = 0.02$, susceptible reference strain vs resistant introgressed strain: $\chi 2 = 2.1$, df = 1, $P = 0.1$).

Fumigation resulted in a significant decrease of 5 to 25% in the numbers of beetles of all strains that were successful in finding the pheromone lure (GLM, $Z = 2.1$, $P = 0.03$) (Table 4.3). After fumigation, the highest and the lowest numbers of beetles successfully locating the pheromone lure were from the field strain and the resistant reference strain, respectively, a relatively similar pattern compared to that before fumigation (Fig. 4.4). The number of beetles that successfully located the lure was not correlated with body mass, neither before fumigation (GLM, $Z = 1.3$, $P = 0.1$) nor after fumigation (GLM, $Z = 0.2$, $P = 0.7$). The routine MR of the insects also had no significant effect on their lure finding ability, neither before fumigation (GLM, $Z = -0.6$, $P = 0.5$) nor after fumigation (GLM, $Z = -1.3$, $P = 0.1$).

4.3.5. Phosphine resistance test for field-cultured beetles

The mortality responses were 100% in groups of field beetles fumigated with the high dose of phosphine and 94% in groups of field beetles fumigated with the low dose of phosphine.

Mortality response to discrimination concentrations of phosphine were interpreted according to Table 4.1.

Table 4.3. Percentage of *Tribolium castaneum* beetles, of different strains, that located the pheromone lure within a given time before and after fumigation, n = 15 for each beetle strain. The significant statistical differences in the percentage of beetles locating the pheromone lures before and after fumigation have been indicated by different superscripts (Chi-square test on raw data).

Strains	Percentage of beetles locating pheromone lure	
	Before fumigation	After fumigation
Field strain	86.5 [a]	60 [b]
Resistant reference strain	26.5[a]	20 [a]
Resistant introgressed strain	40 [a]	20 [b]
Susceptible reference strain	80 [a]	53.5 [b]

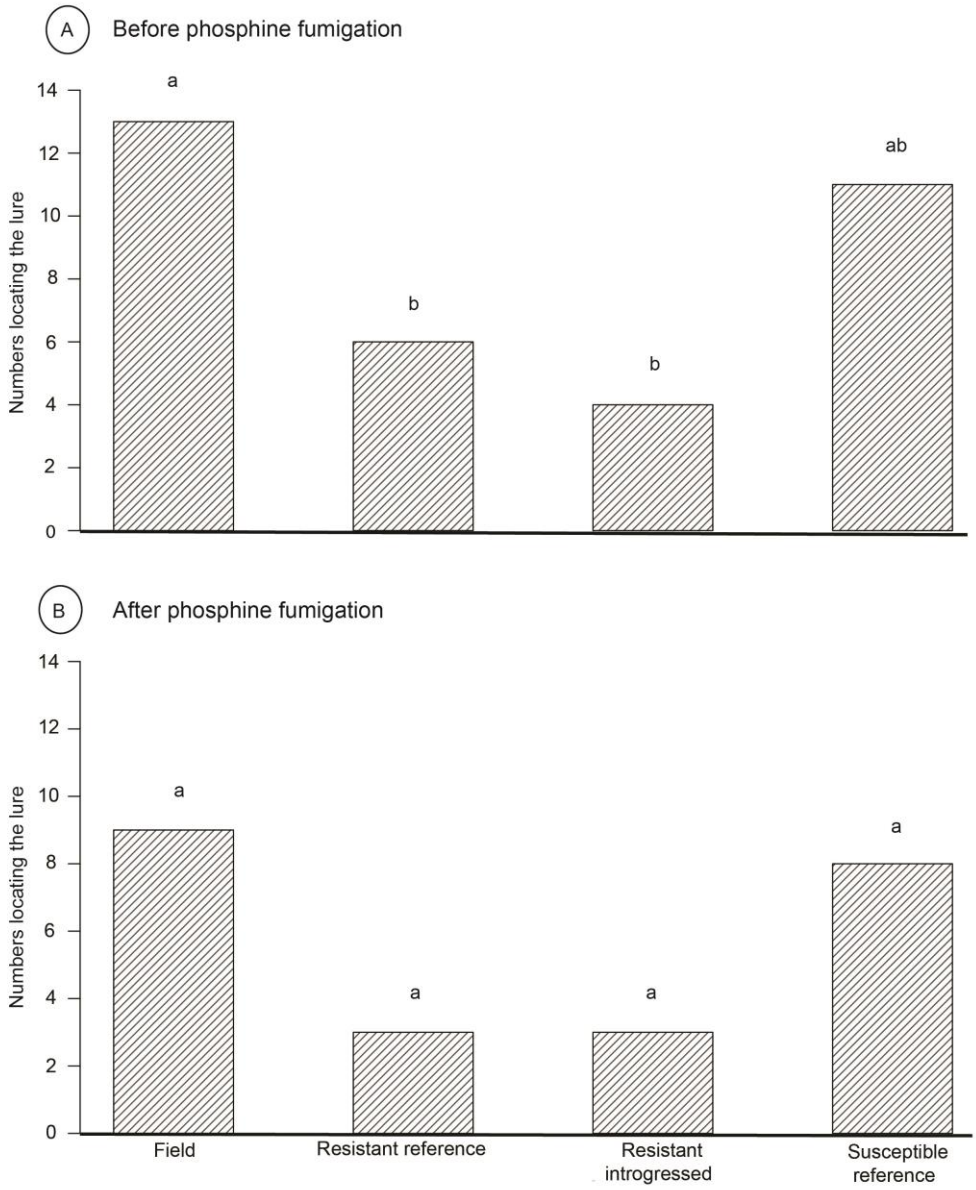

Fig. 4.4. Numbers of *Tribolium castaneum* female adults of a field strain and three laboratory strains that located an aggregation pheromone lure within a given time before fumigation (A) and after fumigation (B), n = 15 for each strain. Results from post hoc pairwise comparisons across the strains are indicated with lower case letters above each bar. Within each of these comparisons, bars that have the same letter are not significantly different from each other.

4.4. Discussion

This study has demonstrated that sublethal exposure to phosphine in combination with the presence of phosphine resistance genes has a significant effect on *T. castaneum* individuals in terms of their metabolic rate, movement levels, and ability to locate a pheromone lure target. These behaviours were assessed before fumigation and 14 days following fumigation. My previous experiments have shown that no changes occurred in the behaviour of beetles in rearing condition over that time frame. Therefore, behavioural changes observed after fumigation are attributable to the fumigation. One field and three laboratory strains including a susceptible reference strain, resistant reference strain and resistant introgressed strain were used in this study. The possible effect of genetic background was controlled by applying the introgression approach (Raymond et al., 2011, Malekpour et al., 2016) and using an introgressed resistant strain that is 98.4% isogenic to the susceptible reference strain. This meant that any differences observed between the introgressed resistant strain and the susceptible reference strain would likely be attributable to the presence or absence of resistance genes.

The association between metabolic rate and phosphine toxicity has been recognized in several studies (Nath et al., 2011; Pimentel et al., 2007). The present study showed that phosphine resistance was consistently associated with routine MR, as susceptible reference strain beetles had a significantly higher metabolic rate compared to that of the laboratory resistant reference beetles (Fig. 4.1A). Allometric scaling predicts that metabolic rate should be correlated with body mass (White, 2011), and it is expected that this scaling relationship should be correlated with differences between resistant and susceptible beetles. The results presented above demonstrated that these predictions were mostly upheld. That is, resistant reference beetles that were smaller than susceptible beetles had lower routine MR (Figs. 4.1 and 4.2) which is

consistent with the predictions of allometric scaling. Routine MR as well as flight propensity and lure locating success was significantly reduced in all strains following fumigation with a sublethal dose of phosphine (Fig. 4.1B). Also, the routine MR of susceptible reference strain beetles was much more inhibited (53.5% inhibition) than that of the resistant reference strain beetles (37.5% inhibition) after fumigation and this inhibition potentially minimized phosphine exposure. These data on the greater inhibition of respiration in the susceptible reference strain is consistent with the findings of Nakakita and Kuroda (1986), who examined both phosphine uptake and oxygen consumption of resistant and susceptible strains of *T. castaneum*. Their results showed that although routine MR of susceptible beetles was more inhibited after fumigation compared to that of resistant beetles, resistant beetles still absorbed more phosphine, which can be related to phosphine absorbance through their integument.

A negative correlation was found between the propensity of flight take-off and the presence of phosphine resistance alleles. That is, laboratory resistant beetles took flight less frequently than laboratory susceptible beetles (Fig. 4.3). The lower propensity of flight initiation in resistant beetles may be a result of their lower routine MR (Fig. 4.1), because Niitepõld et al. (2009) demonstrated that butterflies with higher flight MR flew more than individuals with lower flight MR. A reduction in the flight propensity of all beetles was observed when they were subjected to sublethal doses of phosphine, and a stronger reduction was observed in susceptible reference beetles. The association between walking activity for lure location and phosphine resistance was similar to the association between flight propensity and phosphine resistance genes. That is, a lower number of beetles from the resistant strains were able to locate the lure during the experimental period compared to susceptible reference strain beetles (Fig. 4.4). All four tested populations of *T. castaneum* exhibited decreased success in pheromone lure locating after sublethal phosphine exposure. Exposure to a sublethal dose of

phosphine caused a larger reduction in the percentages of beetles finding the pheromone in susceptible reference strain beetles (26.5% reduction) compared to that of resistant reference beetles (6.5% reduction). The relatively lower reduction in the locomotory responses of resistant strain beetles may indicate that these insects are more able to escape from sublethal phosphine exposure and thus further reduce their phosphine uptake than susceptible beetles. Consequently, the relatively lower reduction in the locomotory responses of resistant strain beetles could increase the potential spread of phosphine resistance when sublethal fumigations are applied.

Although the field strain beetles used in this study were weakly resistant beetles (see results relevant to Table 4.1), the pattern of their movement behaviour and their metabolic rate showed differences compared to the laboratory resistant strains. The laboratory resistant strain beetles were heavier and had a higher metabolic rate, but were less active in both flight and walking behaviour compared to the weakly resistant field beetles (Figs 4.1 to 4.4). These results may imply that short term forces of natural selection have occurred when wild beetles cultured in the laboratory for several generations. Therefore these variations in the phenotype and physiology of laboratory resistant beetles compared to the resistant field beetles can be interpreted as the consequence of adaptations to a new environment.

This investigation provides baseline information for understanding the physiological and behavioural changes of *T. castaneum* following fumigation, especially in resistant beetles. These changes in insect mobility and routine MR may affect the efficacy of phosphine fumigation of stored products and needs to be taken into account when designing phosphine resistance management programmes. Since this study is limited to some extent by the restriction of working in the laboratory, an investigation of the spatio- temporal dynamics at a

relatively large scale in the field (e.g. Ridley et al., 2011) is recommended if pest management programs are to be improved.

Chapter 5

General discussion and suggestions for future research: the relative fitness of resistant *Tribolium castaneum* populations

5.1. Research outcomes

The results presented in this thesis support the argument that activities underpinned by metabolic enzymes can be influenced by the mutations associated with phosphine resistance. The underlying mechanism relates to phosphine resistance having evolved through changes that involve basic metabolic pathways. The nature of this association is discussed below, mainly in the context of fitness costs of other stored grain pests. This forms the basic framework for making suggestions as to future research on *T. castaneum* in relation to its resistance to phosphine.

Results from Chapter 2 showed that the pattern of flight periodicity of resistant strains was relatively similar to that of susceptible beetles (Fig. 2.2). That is, relatively low levels of flight took place in the first half of photophase. A peak in flight initiation was observed at the end of photophase and at the beginning of scotophase (17:00 h in June in Brisbane). It then declined to the lowest level, virtually no flight initiation, and this was maintained until dawn. However, the susceptible strain beetles showed a higher flight propensity, as they took off for flight significantly more frequently than did the resistant strain beetles. Not only did the resistance alleles affect flight propensity, but they also influenced the rate at which resistant beetles walked, the total distance walked, and the angular velocity of walking (Fig. 2.3). That is, the susceptible beetles walked faster and more directly to the test resource (wheat flour), whereas resistant beetles walked more slowly over a significantly longer distance, and therefore fewer of them located the resource during the allocated test period (Table 2.2). The prediction is that resistant beetles in the field will fly less, and when they do fly, are significantly less likely to locate suitable resources than will susceptible beetles.

These findings from Chapter 2 are the first to indicate a negative association between phosphine resistance genes and the movement abilities of *T. castaneum* beetles. I conclude from these results that impairment of the locomotory abilities of phosphine resistant beetles is likely to have consequences that influence the development and spread of resistance alleles spatially among populations of *T. castaneum*. However, these conclusions need to be investigated further in the field, where research on the walking and flight responses of wild resistant populations of *T. castaneum* will undoubtedly extend our understanding of their population ecology and thus the spread of phosphine resistance.

Chapter 3 demonstrated that the presence of odour stimuli of various types resulted in faster and more frequent flight initiation of beetles, of resistant and susceptible strains, but had no significant effect on the direction and speed of flight (Fig. 3.2 bottom). All the beetles of both strains flew in the direction of the wind, as they did in the absence of odour stimuli. Cotton seeds and *T. castaneum* pheromone lure were the most attractive of the test attractants for *T. castaneum* beetles, of both susceptible and resistant strains. The presence of an odour stimulus also resulted in faster and more successful food location by beetles, of both resistant and susceptible strains. Odours from pheromone lures and cotton seeds attracted significantly more beetles in a shorter period than the other odours tested, and this was true of both resistant and susceptible strain beetles (Fig. 3.4 middle). Therefore the attraction of susceptible and phosphine resistant *T. castaneum* beetles in response to odours of various types, and also their behaviour associated with the localization of those odours, were similar to one another.

The results from wind tunnel assessments, presented in chapter 3, also indicate that mating status had no significant effect on the flight propensity of both susceptible and resistant

beetles, because nearly similar numbers of susceptible and resistant strain beetles initiated flight (Fig. 3.3). The flying beetles, whether mated or unmated, also took nearly equal times to initiate flight and also maintained almost identical flight speed. The mated beetles of both susceptible and resistant strains were significantly less attracted towards the lures compared with unmated beetles of those strains, as indicated by their taking longer to locate the lure (Fig. 3.5). After mating, therefore, the walking activity of these beetles is more prone to change than is their flight intension, regardless of the presence of phosphine resistance genes.

The main conclusions from Chapter 3 are that both environmental and intrinsic factors had similar effects on the movement ability of both phosphine resistant beetles and susceptible strain beetles when these insects seek and colonize the resources in their patchily distributed environment. Such studies will not only provide insight into a full understanding of the pleiotropic effects of phosphine resistance genes on the flight and resource location of *T. castaneum* beetles, but may have important practical implications. For example, the development of food-baited trapping systems, which will undoubtedly be useful for the environmentally safe management of pests, needs to be done with these points in mind. Also, this knowledge is likely to be relevant to the development of effective resistance management strategies, especially with respect to reducing frequencies of resistance alleles in the field.

The results presented in Chapter 4 revealed the physiological impacts of sublethal exposure to phosphine on resistant *T. castaneum* relative to susceptible ones. All beetles suffered a reduction in flight initiation rates and a decrease in the successful location of aggregation pheromone lures (Figs. 4.3 and 4.4). The hypothesized reason for the reduced locomotory responses following exposure to phosphine was a decrease in the metabolic rate of the beetles. Results from this study support the hypothesis. That is, the exposure to sublethal

doses of phosphine reduced routine MR of both susceptible and resistant beetles. The susceptible beetles showed a significantly greater reduction in CO_2 production after exposure to sublethal doses of phosphine compared to the resistant beetles, although the routine MR was still higher in susceptible beetles. Simultaneously, much larger decreases in the activities associated with both walking and flight were observed in the susceptible beetles, when exposed to sublethal doses of phosphine, compared to the decreases recorded in resistant beetles (Tables 4.2 and 4.3).

The findings presented in Chapter 4 therefore suggest a new perspective on how to evaluate the survival strategies of resistant and susceptible beetles of *T. castaneum* when exposed to sublethal doses of phosphine, a situation that is frequent in most parts of the world because fumigation practices tend generally to be poor (Boxall, 2001). My conclusion is that the relatively greater reduction in the locomotory responses of susceptible beetles showed that these insects may withstand exposure to phosphine by remaining relatively still and reducing their routine MR as much as possible. They would thus minimize their exposure to phosphine, perhaps only incidentally. By contrast, phosphine resistant beetles of this species, when exposed to phosphine fumigation, may continue to move and incidentally remove themselves from areas of phosphine exposure. These results have implications for the aim of promoting improved resistance management of stored grain pests.

Finally, the results from field beetles (which are, essentially, field-collected beetles cultured for only one or two generations), presented in chapters 2 to 4, indicate that the wild population of *T. castaneum* beetles has differences in their movement propensities and characteristics compared to those of the beetles cultured in the laboratory over numerous generations. In particular, the field beetles showed stronger attraction to odours of various

types. Because they walked faster and more directly and had to cover significantly less distance to reach the food and pheromone lures compared to laboratory beetles (Figs 2.3B and 2.3C). Field beetles also had a higher flight propensity compared to the laboratory beetles (Figs 2.2, 3.1 and 4.3A). Culturing these insects over many years has led to an increase in the metabolic rate of individual adults, since field beetles produced significantly lower amounts of CO_2 in respirometry tests (Fig. 4.1A). The difference in routine MR across field beetles and laboratory beetles may result from the differences in their body mass, as field beetles were also much lighter than laboratory cultured beetles (Fig. 4.2). These results support the hypothesis of differences between the biological characteristics of field and laboratory populations, and it is likely they differ in other aspects as well (Ahmad et al., 2012, Aulicky et al., 2015, White, 1984, Toro et al., 1979). The differences may result from unintentional selection imposed over successive generations on the beetles in the laboratory cultures. These insects exist in an environment of high quality food resources, which requires no dispersal and no need to locate resources, and potential mates are constantly nearby. Although the differences in the behaviour of laboratory and field beetles need to be explored further, it is highly recommended to use field beetles in behavioural and ecological studies because of the significantly different performance of field beetles compared to the laboratory beetles. Presumably these differences mean that field beetles yield results that are more realistic, and therefore more relevant to understanding the ecology of these insects.

The results and conclusions from Chapters 2 to 4 indicate that negative pleiotropic effects of phosphine resistance alleles result in fitness costs and would likely have consequent effects on energy demanding activities, such as dispersal and resource location of these beetles. The literature on the pleiotropic effects of phosphine resistance (which is mainly restricted to stored-product pests among insects) is largely anecdotal and scattered, as well as somewhat

contradictory. The following section therefore reveals the resistance status of other major stored-product pests and reviews the fitness costs they suffer as a resulted of the pleiotropic effects of phosphine resistance genes. This section aims to provide a stronger basis for the investigation and understanding of the differences in the physiological and behavioural responses of resistant *T. castaneum* beetles relative to susceptible ones, through comparison with the equivalent responses in other stored-product pests.

5.2. Fitness costs of resistant insects

An overview of the literature on the fitness costs imposed on individuals from populations of stored-product insect pests with insecticide resistance is presented in Table 5.1. The genetic basis of insecticide resistance means that pleiotropic effects are likely to influence the fitness of the insects carrying resistance genes when these insects are in an environment without exposure to insecticides (Mckenzie, 1996). Individuals carrying resistance alleles are generally less fit relative to their susceptible counterparts when they are not in the presence of the selective agent. A large number of fitness studies has shown that various life process and reproductive outputs of organisms are negatively affected by resistance genes, as indicated by the cases summarized in Table 5.1. For example, Pimentel et al. (2007) investigated the fitness costs associated with insecticide resistance in populations of the stored-product pest beetles *T. castaneum*, *Rhyzopertha dominica* and *Oryzaephilus surinamensis* and found a negative association between resistance genes and the reproduction and the reproductive rate of these insects. These resistant populations also had slower developmental and population growth rates than their susceptible counterparts (Sousa et al., 2009). Pimentel et al. (2012) reported a negative relationship between the level of phosphine resistance and walking behaviour of *R. dominica*, as more resistant strains walked significantly less. Nevertheless, evolutionary processes may be able to modulate the fitness costs to resistant insect, and even

perhaps confer a fitness benefit to them, through the agency of modifier genes or through replacement of the resistance allele by a less costly allele that is able to mitigate the cost of insecticide resistance (McKenzie ,1996). In line with this suggestion, some studies that measured fitness found a neutral effect of the resistance genes (neither a fitness cost nor a fitness benefit) on different aspects in the life of the affected organisms.

Clarification of the direction in which phosphine resistance alleles affect the fitness (whether a fitness cost or benefit) of the pests concerned is directly relevant to considerations related to the development of effective resistance management strategies (Lockwood et al., 1984). That means that for resistant populations in which insecticide resistance is associated with a fitness cost, the appropriate management strategy could be withholding insecticide exposure for a long period to reduce phosphine resistance development in resistant populations (Pimentel *et al*. 2007; Sousa *et al*. 2009). However, for resistant populations in which phosphine resistance confers a fitness benefit (as a result of modifier genes or their substitution by less costly genes), an effective management strategy would be provided by infrequent use of insecticide. Therefore knowledge of the fitness consequences of resistance alleles will help in designing efficient insecticide resistance management programs. Despite the importance of fitness traits with respect to resistance management, this concept remains largely unstudied in phosphine resistant pest populations. Fitness studies are not only important for the development of appropriate pest management plans, but also are helpful in evolutionary studies of newly adapted phenotypes and the related changes they impose on the physiology of the resistant population.

Table 5.1. A survey of the literature on the fitness costs imposed on individuals of stored-product beetle pests with insecticide resistance compared to their susceptible counterparts.

Insect species	Biological parameter	Direction of the imposed fitness			Insecticide	Reference
		Fitness cost	Fitness benefit	Neutral		
Tribolium castaneum	Fecundity	Lower oviposition rate and fecundity			Phosphine	Saxena & Bhatia, 1980, Pimentel et al., 2007
	Respiration rate	Lower respiration rate			Phosphine	Pimentel et al., 2007
	Developmental rate	Slower developmental and population growth rates			Phosphine	Sousa et al., 2009, Kaur et al., 2012
	Fecundity, egg-fertility & developmental rate		Higher fecundity	No difference in egg fertility and development time compared with susceptible beetles	Malathion	Arnaurd et al., 2002, Haubruge & Arnaud., 2001
	Reproductive success		Greater reproductive success in resistant males		Malathion	Arnaud & Haubruge, 2002

[Continued]

Insect species	Biological parameter	Direction of the imposed fitness			Insecticide	Reference
		Fitness cost	Fitness benefit	Neutral		
	Development time		Longer development time		Malathion	Haubruge & Arnaud., 2001
	Walking and flight parameters	Less flight initiation, and less successful in resource location			Phosphine	Malekpour et al., 2016
Rhyzopertha dominica	Respiration and reproductive rates	Lower growth rate and respitration rates			Phosphine	Pimentel et al., 2007
	Locomotion rate			No significant difference in duration of walking compared with susceptible beetles	Phosphine	Kaur et al., 2013
	Locomotion rate	Lower rate of walking			Phosphine	Pimentel et al., 2012

[Continued]

Insect species	Biological parameter	Direction of the imposed fitness			Insecticide	Reference
		Fitness cost	Fitness benefit	Neutral		
Oryzaephilus surinamensis	Respiration and reproductive rates	Lower respitration and growth rates			Phosphine	Pimentel et al., 2007
	Allele frequency in culture	Faster decrease in percentage of resistant adults in a given period			Malathion	Muggelton, 1983
Sitophilus zeamais	Development and reproductive rates	Lower development rate and population growth in resistant population from Juiz de Fora, Brazil		Similar development rates and population growth in resistant population from Jacarezinho, Brazil, compared with susceptible ones	Pyrethroid	Fragoso et al., 2005
	Enzyme activity	Enhanced activity of carbohydrate, lipid-metabolizing, proteolytic and cellulolytic enzymes			Pyrethroid	Araújo et al., 2008a, b

[Continued]

Table 5.1 Continued

Insect species	Biological parameter	Direction of the imposed fitness			Insecticide	Reference
		Fitness cost	Fitness benefit	Neutral		
	Size of fat body cells		Larger fat body cells		Pyrethroid	Guedes et al., 2006
	Body mass and respiration rate		Higher body mass and respiration rate of the resistant populations from Jacarezinho, Brazil		Pyrethroid	Oliveira et al., 2007, Guedes et al., 2006
	Fluctuating asymmetry		Lower fluctuating asymmetry		Pyrethroid	Ribeiro et al., 2007
	Respiration rate	Lower respiration rate			Phosphine	Pimentel et al., 2009
Cryptolestes ferrugineus	Life span		Longer life span		Malathion	White & Bell, 1995

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5.3. Future directions

Based on the findings derived from Chapters 2 to 4 and the literature survey provided in Table 5.1, the following aspects of the ecology of *T. castaneum* are suggested as priorities in developing a fuller understanding of the effects of phosphine resistance genes on the ecology of *T. castaneum* and thus the spatio-temporal dynamics of resistance in this (and other) species.

The pleiotropic effects associated with phosphine resistance genes have the potential to influence negatively different aspects of the movement of *T. castaneum* in the absence of phosphine exposure. Less directed orientation and a longer time taken to detect and locate food resources in resistant beetles could be related to a decrease in the sensitivity of the olfactory receptors of phosphine resistance individuals (Foster et al., 1999). That is, the pleiotropic effects of phosphine resistance alleles result in a weakening of the ability of phosphine resistant beetles to screen ecologically significant chemicals with their antennal receptors, and thus weaken the ability of these insects to forage. To test this proposal, I suggest a comparison of the antennal responses of resistant and susceptible beetles through electroantennography and related experiments.

A fuller knowledge of the effects of phosphine resistance alleles on the walking activity of *T. castaneum* can be achieved by examining all motile life stages of these insects. Such information will show how the expression of pleiotropic effects of resistant genes varies through the life of the beetle (Kaur et al., 2012). Thus for the development of more effective resistant management programs, I recommend further investigation on the consequent effects of phosphine resistance alleles on the walking activity of *T. castaneum* larvae (the only motile stage in the life of *T. castaneum*, besides the adult stage).

This study has been restricted to short-range resource localization experiments conducted within the laboratory, but it has generated predictions as to how resistant insects are likely to be affected in the field with respect to long distance movement (e.g. as recorded by Campbell et al., 2002 and Ridley et al., 2011). To test these predictions, temporal and spatial studies in the field should be the next priority in resistance studies of *T. castaneum* beetles. In other words, there is a need for conducting fitness studies in the field.

Also, in this thesis the behaviour of laboratory and wild beetles has been compared and results show conclusively that culturing these insects over many generations under laboratory conditions changes the behaviour of these beetles enough to affect interpretations of their ecology in the field. Therefore, future studies should be based on beetles from wild populations to ensure that management programs relevant to this globally serious pest are based on the most realistic interpretations of its ecology.

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Appendices

Appendix a:

The following appendices are the supporting information for the results included in Results Chapter 3.

Appendix 3.1. Results of post hoc pairwise comparisons of the flight direction of *Tribolium castaneum* adults in response to different resources.

Test lures compared	Z value	*P* value
Cotton-control	0	1
Pheromone-control	0	1
Wheat flour-control	1.2	0.5
Pheromone-cotton	0	1
Wheat flour-cotton	1.2	0.5
Wheat flour-pheromone	1.2	0.5

Appendix 3.2. Results of post hoc pairwise comparisons of the flight direction of *Tribolium castaneum* adults with respect to their bearing phosphine resistance genes.

Strains	Z value	*P* value
Field vs Resistant introgressed	-1.2	0.5
Field vs Resistant	-1.2	0.5
Field vs Susceptible	-1.2	0.5
Resistant vs Resistant introgressed	0	1
Susceptible vs Resistant introgressed	0	1
Susceptible vs Resistant	0	1

Appendix 3.3. Results of post hoc pairwise comparisons of the flight speed of *Tribolium castaneum* adults in relation to their exposure to different resources.

Test resources compared	Z value	*P* value
Cotton-control	-0.9	0.78
Pheromone-control	0.1	1
Wheat flour-control	-0.3	0.98
Pheromone-cotton	1.3	0.72
Wheat flour-cotton	0.5	0.93
Wheat flour-pheromone	-0.4	0.96

Appendix 3.4. Results of post hoc pairwise comparisons of the flight speed of *Tribolium castaneum* adults in relation to the presence of phosphine resistance genes.

Strains	Z value	*P* value
Field vs Resistant introgressed	-25.3	<0.001
Field vs Resistant	-25.6	<0.001
Field vs Susceptible	-14.4	<0.001
Resistant vs Resistant introgressed	-0.3	0.98
Susceptible vs Resistant introgressed	11.8	<0.001
Susceptible vs Resistant	12.1	<0.001

Appendix 3.5. Results of post hoc pairwise comparisons of the time taken to initiate flight by *Tribolium castaneum* adults in relation to their bearing phosphine resistance genes.

Strains	Z value	*P* value
Field vs Resistant introgressed	23.3	<0.001
Field vs Resistant	23.6	<0.001
Field vs Susceptible	16.05	<0.001
Resistant vs Resistant introgressed	0.3	0.9
Susceptible vs Resistant introgressed	-8.5	<0.001
Susceptible vs Resistant	-8.8	<0.001

Appendix b:

A joint-paper has been prepared and submitted to an international peer reviewed journal, entitled Entomologia Experimentalis et Applicata. on a topic related to the results presented in this thesis. It was envisaged to be part of a collaboration with Pieter Arnold and that work would have amalgamated our respective methodologies into an evolutionary study related to beetle migration, but the collapse of cultures prevented these plans being put in place.

Investigating movement in the laboratory: dispersal apparatus designs and the red flour beetle, *Tribolium castaneum*

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Short title: Investigating *Tribolium* movement in the laboratory

Abstract

The natural dispersal of *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) has been emulated in the laboratory for more than 50 years, using a simple dispersal apparatus. This has typically comprised of a starting container (initial resource or patch) connected by tubing, which contains thread for the animals to climb into a tube and hence to an end container. That is they move to a new viable resource or patch from an inter-patch zone or non-viable habitat. We modified this basic apparatus design to test the effect of tubing length and tubing insertion angle on the dispersal rate and proportion of successful dispersers. We expected that the proportion of successful dispersers would be repeatable within each apparatus design, and that increasing tubing length and steepness of the insertion angle would reduce dispersal rate and success across apparatus designs. Dispersal increased linearly through time, similarly so for both males and females. The design with the most vertical tubing insertion angle had a lower proportion of successful dispersers. Tubing length also had a negative relationship with dispersal success (as judged by insects reaching the end container), but a significant reduction in dispersal success was only apparent between the shortest and longest tubing between containers. We suggest that locating and climbing the vertical section of string before they can enter the tubing between containers restricts dispersal and that at higher densities insects exhibit greater inclination to climb. This type of apparatus has flexible design tolerances and further potential to study the dispersal of other small insect species that primarily use pedestrian locomotion.

Keywords: disperser, emigration, immigration, patch, rate of spread, resident, Tenebrionidae

Introduction

Flour beetles of the genus *Tribolium* (Coleoptera: Tenebrionidae), particularly *T. castaneum* Herbst and *T. confusum* Duval, are major pests of a wide range of grain species and processed stored products globally, and are known to have a high rate of movement among resource patches (Ahmad et al., 2012; Campbell & Hagstrum, 2002). A diverse array of approaches have been used to study the movement of *Tribolium* beetles including laboratory apparatuses (e.g. Łomnicki, 2006; Prus, 1963), warehouse level patch exploitation arenas (e.g. Campbell & Arbogast, 2004; Campbell & Hagstrum, 2002), and landscape scale sampling (e.g. Ridley et al., 2011). Here we focus on movement in apparatuses. Most apparatus designs consist of connected containers that adult beetles can move between, allowing dispersers and non-dispersers to be separated over time. The main phases of successful dispersal can be observed with this design; the inclination to move away from a resource patch (initial container), survival through an unsuitable inter-patch zone (tubing between patches, or in intermediate containers of various suitabilities), and establishment into a new patch (final container) (Bowler & Benton, 2009). While this has been the most widely used approach, little consensus has emerged on certain apparatus design attributes.

Prus (1963) described an apparatus to study the tendency for beetles to emigrate from a vial containing flour (*A*) to an empty vial (*B*) by climbing cotton thread into a tube. The tubing was inserted through the vial lids (bridging the two vials) and the thread, which dangled onto the surface in *A* but not in *B*, permitted only one-way movement from *A* to *B*. Emigration rate of beetles from *A* to *B* was repeatable over 10 days and multiple experimental replicates (Prus, 1963). Despite inexplicit design specifications (tubing internal diameter; 4.5 mm, and initial density: 32 beetles per 8 g of flour), the repeatability of the recorded behaviour and design simplicity ensured its longevity in subsequent studies of *Tribolium* dispersal.

Modifications to the Prus design such as that of Ogden (1970a); b) included flour medium in the second vial, and also defining the length, shape and material of tubing (Tygon tubing, U shaped and 150 mm in length), container size (29.5 ml), and population density (either 32 or 50 individuals per 8 g of flour). Ziegler (1976) utilised pipe cleaner, in place of thread, and glass tubing, but described no other design parameters.

The Prus design was used as a platform for investigating *Tribolium* emigration in response to numerous factors, including population density, age, artificial selection for activity or emigration, and also fecundity and life-history responses to selection for emigration (Ben-Shlomo et al., 1991; Goodnight, 1989; Lavie & Ritte, 1978; 1980; Ogden, 1970a; b; Prus, 1966; Riddle & Dawson, 1983; Ritte & Lavie, 1977; Ziegler, 1976; 1977; Zirkle et al., 1988; Zyromska-Rudzka, 1966a; b). Ritte and Lavie (1977) induced divergent selection on dispersal in just one generation by selecting for beetles that moved from vial *A* to *B* (via a 30 cm long polyvinyl tube), twice in two opportunities, as high dispersers and those that did not move as low dispersers. Łomnicki (2006) used a one-way dispersal apparatus including five beakers of various size (*A*–*E*), three of which contained flour (*A*, *C*, *E*) and two of which were unsuitable habitats (i.e., empty: *B*, *D*), all connected by glass tubing containing string. Dispersal through this apparatus was far slower than previous designs, suggesting that the extra beakers limited successful dispersal rate.

Despite the differences among designs, many of these studies reached similar conclusions. Emigration tendency was low in immature individuals, peaked around sexual maturity, and declined later in life. Males emigrated more rapidly than females when the sexes were kept separately, and keeping the sexes mixed resulted in an overall emigration rate that was intermediate between those of the sexes separately (Ogden, 1970b; Prus, 1963; Riddle &

Dawson, 1983; Ziegler, 1976). Dispersal was dependent on density and age of infestation; this was thought to be a response of repulsion to flour that was "conditioned" by chemical secretions and frass accumulation over time (Ogden, 1970b; Zyromska-Rudzka, 1966a). A discernible difference across the emigration rates of lines selected for dispersive and non-dispersive behaviour was identified after five generations of selection (Ogden, 1970a) and dispersal behaviour has an underlying genetic component (Lavie & Ritte, 1978; Riddle & Dawson, 1983; Ritte & Lavie, 1977).

Studies on *Tribolium* dispersal in the laboratory have almost ubiquitously used the apparatus design of Prus (1963) with modifications to the container size, tubing length and material, container arrangement, population density, and time period. However, many studies have not provided detailed specifications of apparatus components. The length of tubing between containers (which represents the inter-patch dispersal component), the angle at which the tubing is inserted into container lids (which may increase dispersal difficulty) are unstated, or vary significantly among studies. The importance of these factors to dispersal success has not been investigated previously. In the present study we have implemented and tested design aspects derived from apparatus revisions by addressing the following questions: 1) does increasing tubing length affect the proportion of successful dispersers and the dispersal rate; 2) does tubing insertion angle affect the proportion of successful dispersers?; and 3) is dispersal rate repeatable within an apparatus design? We predicted that longer tubing and more vertical tubing insertion angle would reduce the proportion of successful dispersers. We addressed these questions using *T. castaneum* and five dispersal apparatus designs, while manipulating tubing insertion angle and length.

Materials and methods

Animals and housing

A wild-type population of *T. castaneum* (QTC4) was sourced from the Postharvest Grain Protection Team (Department of Agriculture, Fisheries and Forestry; Brisbane, QLD, Australia) and used to establish experimental stocks. The QTC4 strain originated from a storage facility in Brisbane (QLD, Australia) in 1965. It has been cultured ever since in the absence of selective pressures from the fumigant phosphine, and therefore these insects exhibit natural susceptibility to phosphine. Stocks were maintained on 210 g of flour medium containing wholemeal stoneground wheat flour (Kialla Purefoods; Greenmount, QLD, Australia) and torula yeast (Lotus Foods Pty. Ltd., Cheltenham, VIC, Australia) at a ratio of 19:1, in 1 l cylindrical containers at 29.5±1 °C and room humidity (moderate). Stocks were cultured fortnightly to maintain clean housing and separate cohorts. Beetles used in experiments were collected from stocks as pupae, and sex was determined by examining the external genitalia (Halstead, 1963) under an Olympus SZ61 stereomicroscope (Olympus Australia Pty. Ltd.; Notting Hill, VIC, Australia). After sorting by sex, pupae were randomly added to 70 ml containers that held 15 g of flour medium in groups of 50, such that each experimental replicate had five containers (i.e., 250 male and 250 female pupae, and a total of 3000 individuals over six experimental replicates). They were held for six days to allow the resultant adults to reach three days of age post-eclosion before the 70 ml containers were attached as container *A* in the dispersal apparatus, for the dispersal experiments to commence.

Dispersal apparatus designs

Five designs were chosen to test the dispersal capacity of *T. castaneum*, based mostly on the designs of Prus (1963), Ogden (1970a) and Łomnicki (2006). Variables that were manipulated in this study were the length of the tubing and the angle of the tubing as it left

and entered containers. Each design used three 70 ml containers (57 × 44 mm, labelled *A*, *B* and *C*, respectively; Sarstedt Australia Pty. Ltd., Mawson Lakes, SA, Australia) connected through the lid of each container via silicone tubing (4 mm internal diameter), containing a single looped strand of cotton twine that permitted only one-way movement from container *A* to *B* to *C*. As the angle of tubing was negatively correlated with the distance between containers, only Designs 1 and 2 were compared to determine the effect of tubing insertion angle independently of length. Tubing length in Design 1 was 140 mm, with a distance of 70 mm between the tube insertions, yielding a relatively steep insertion angle (55°) for the tubing (Figure 1A) to represent the extended vertical climbing distance employed in the design of Prus (1963). Design 2 used the same tubing length as Design 1 (140 mm) over a greater distance between the tube insertions (120 mm), creating a shallower slope (24°) in the tubing (Figure 1B). Designs 3, 4 and 5 all had relatively shallow tubing insertion angles (4–16°); these designs were included to test if increasing tubing length reduced dispersal success. Design 3 used 185 mm long tubing over 165 mm (Figure 1C), Design 4 used 335 mm long tubing over 310 mm (Figure 1D), and Design 5 employed 640 mm long tubing over a distance of 620 mm between tubing insertions (Figure 1E). The distances between insertion points in containers in the different dispersal apparatuses were structurally maintained using plywood housing to fix the containers a set distance apart, level with each other and aligned linearly (Figure 2).

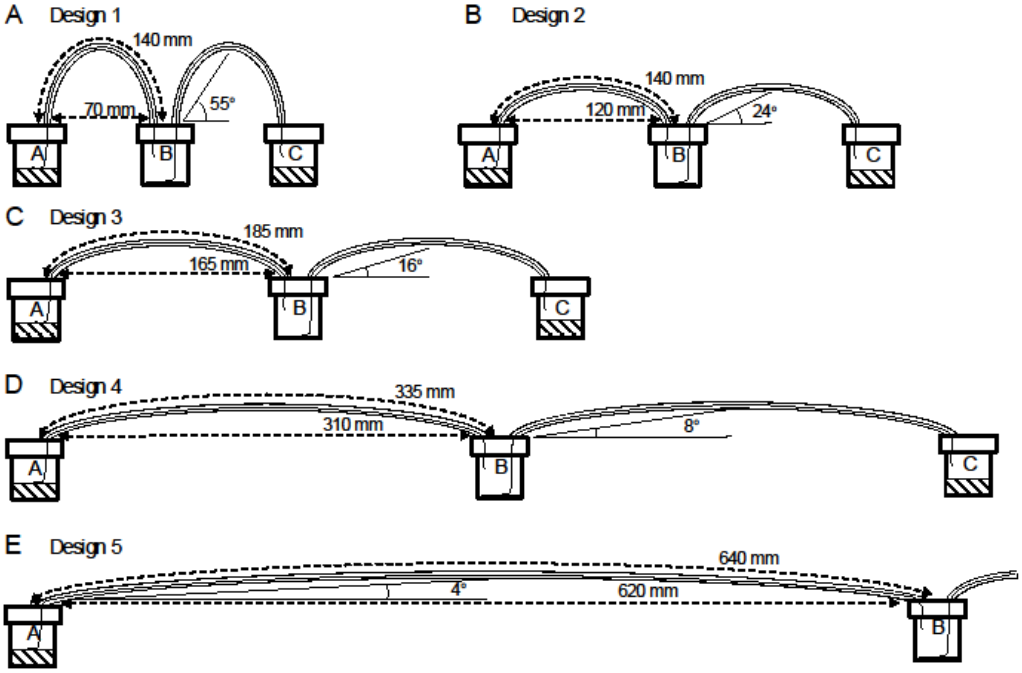

Fig. 1. Dispersal apparatus designs used to investigate the effects of tubing angle and length on the dispersal success of adult *T. castaneum*. Each design used three containers (A, B & C) connected by tubing containing string for beetles to climb, allowing one-way movement from A–B–C. (A) Design 1; 55° tubing insertion angle between containers over 140 mm tubing length, (B) Design 2; 24° tubing insertion angle over 140 mm tubing length, (C) Design 3; 185 mm tubing length, (D) Design 4; 335 mm tubing length, and (E) Design 5; 640 mm tubing length. Design 5 B–C is identical to A–B but not shown due to its size. Curved dashed arrows show tubing length; straight dashed arrows show distance between insertion points, and angles show the approximate angle from the point of insertion to the maximum height of the tubing over the distance between insertion points.

Dispersal assessment

Each design used two apparatuses, one for males and one for females, to assess dispersal for each sex separately but concurrently. Sexes were not mixed for two reasons: to eliminate the potential for breeding to occur during the dispersal process, which has been shown to slow the rate of dispersal (Ziegler, 1976) and to emulate the conditions required for a subsequent experiment that controlled breeding after the dispersal assessment. The containers with 50 adult beetles of known sex, labelled container *A*, were randomly assigned to an apparatus design and attached to each apparatus, representing the starting point of dispersal (Figure 2). Both *A* and *C* contained 15 g of flour and container *B* had a covering of paper to provide grip, but was otherwise an unsuitable habitat for the beetles. Container *B* was included to represent a patch that would not be a suitable resource to establish in, but that had to be passed through as part of the dispersal process (Łomnicki, 2006).

Dispersal apparatuses were placed in a controlled temperature room with identical conditions to the stock populations and apparatus position was randomised at the beginning of each experimental replicate. Once container *A* was connected to each of the apparatuses, dispersal assessment commenced and counts were made during 0830–0930 and 1630–1730 h daily for 96 h (nine counts). Counts of beetles were recorded in the connecting tubes (*A*–*B* and *B*–*C*) and in containers *B* and *C*. Both sets of tubing and container *B* could be counted visually without disturbing the apparatus, however container *C* required detachment. Flour was carefully tipped into a container and gently swept with a paintbrush to draw beetles to the flour surface for counting. Flour and beetles were then funnelled back into container *C* and reattached to the apparatus. Container *A* was left undisturbed throughout the experiment to facilitate natural dispersal through the apparatuses. The number of beetles in container *A* was calculated at each time point by subtracting the total number of beetles in all other containers

and tubing from the starting total of 50. Mortality was assessed at the conclusion of each experiment, but was negligible (two adults at most in any given replicate). The assessment was repeated six times, each with a different cohort of beetles.

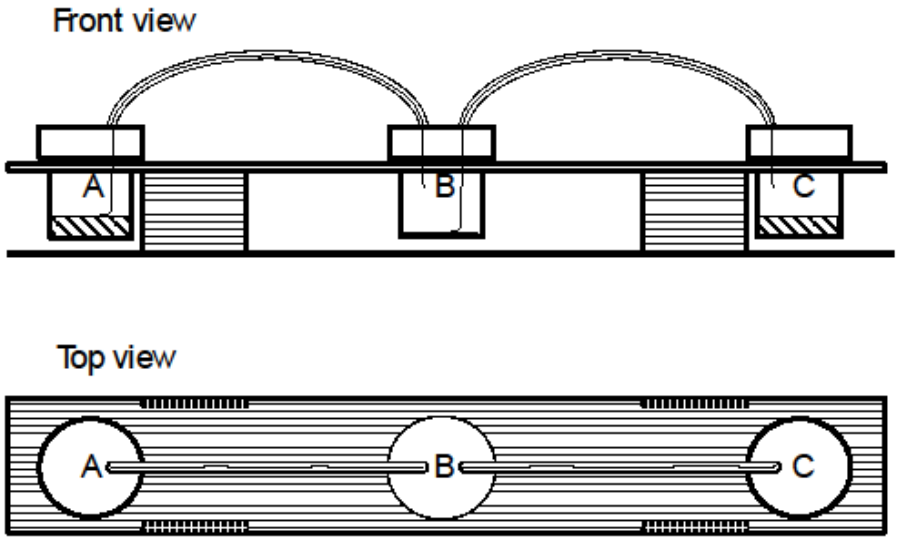

Fig. 2. Example schematic of Design 3. Front and top views of the plywood housing for maintaining structure and consistency of the tubing insertion angle and tubing length for the dispersal apparatuses.

Statistical analyses

Data were treated as proportional data, where the principle variable of interest was proportion of beetles reaching container *C* (successful dispersers). Fixed factors were apparatus design, tubing length, insertion angle of tubing, sex, and time. Replicate (cohort) was treated as a random factor in all analyses. A full model was fitted using a generalised linear mixed effects model with a binomial error structure and logit link function from the lme4 package (Bates et al., 2014) in R 3.2.3 (R Development Core Team, 2015). Models were simplified by

removing non-significant interaction terms and using ANOVA and Akaike information criterion (AIC) to compare the resulting simplified models. Data presented are mean proportions of beetles reaching container *C* ± SEM, and α was set at 0.05.

Results

Dispersal rates and success

The proportion of beetles that reached container *C* at any given time was relatively consistent across all designs and between sexes, where males are presented separately to females (Figure 3). The proportion of successful dispersers increased significantly with time and was repeatable, following a near-linear trajectory to about 60–80% successful dispersal after 96 h (Figure 3). Time was positively correlated with the proportion of successful dispersers and was highly significant for all apparatus designs, but no difference between male and female beetles was detected (Table 1). Design 2 was chosen as the reference apparatus against which the other designs were compared as it had the shortest tubing with a shallow insertion angle (Table 1). This design was predicted to, and did, yield the fastest dispersal rates in both sexes. Only Design 5 had a significantly lower proportion of successful dispersers than Design 2, whereas all other designs were not significantly different from this design or from each other (Table 1).

Table 1 Generalised linear mixed effects model (GLMM) of the effect of time, design and sex on the proportion of beetles successfully dispersing from *A* to *C*. Designs are all compared to reference Design 2 (short tubing length and shallow insertion angle).

	Estimate	SE	Z-value	P-value
Intercept	-3.788	0.187	-20.3	<0.001
Time (hours)	0.555	0.001	72.11	<0.001
Design 1	-0.412	0.233	-1.76	0.078
Design 3	-0.321	0.233	-1.38	0.168
Design 4	-0.42	0.233	-1.8	0.072
Design 5	-0.565	0.234	-2.42	0.016
Sex	0.206	0.148	1.39	0.164

Random effect: replicate (intercept) variance = 0.311, std. dev. = 0.558

Tube length

Increasing tubing length between containers had a negative relationship with the proportion of successful dispersers (i.e., as tubing length increased from 140 mm in Design 1 to 640 mm in Design 5, the regression slope decreased; Table 1). The proportion of successful dispersers was lowest in Design 5, where tubing length was greatest between containers, and this was significantly different to Design 2 (Table 1). Effectively, the greater the distance between containers, the longer it takes the beetles to move between them. Therefore the overall proportion of beetles in container *C* at the end of the experiment was lower than the designs with shorter tubing between containers (Figure 3).

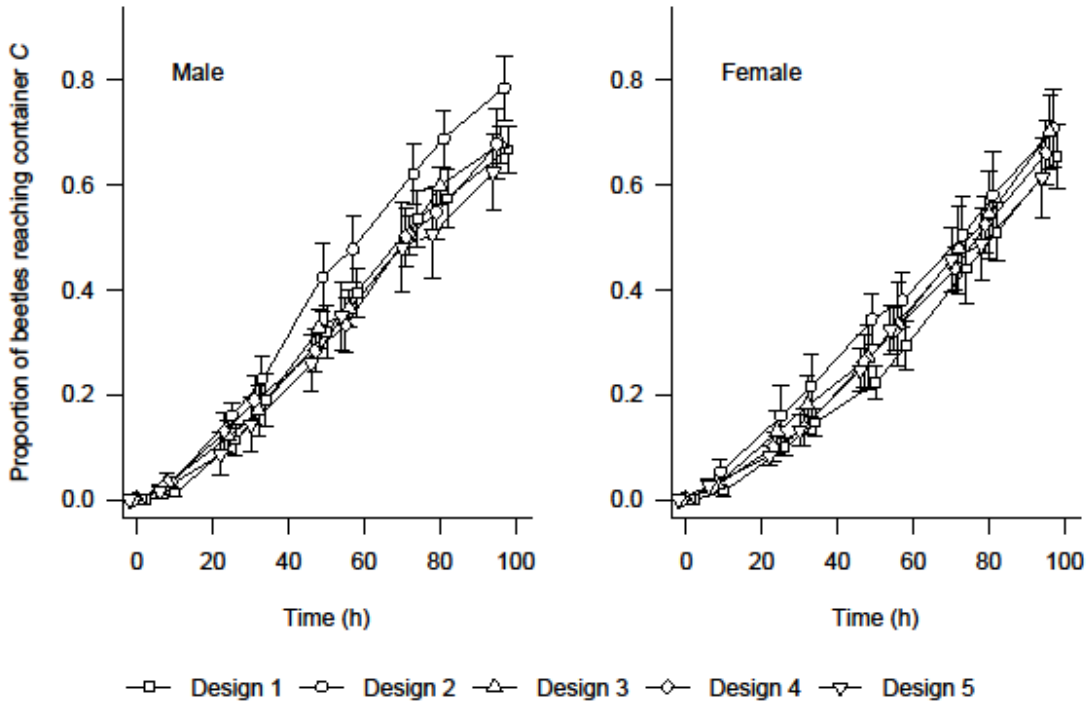

Fig. 3. Mean proportion of male and female *T. castaneum* adults reaching the final container *C* over time across the five apparatus designs. Data are presented as means ± SEM from six independent replicate cohorts.

Tubing insertion angle

The angle that tubing projected from the lids of each apparatus container was predicted to have a significant effect on the proportion of successful dispersers, by increasing the difficulty of dispersal thereby reducing the attainable dispersal rate. Designs 1 and 2 were directly comparable with respect to determining the effect of insertion angle independent to tubing length, therefore these were the only designs included in the model (Table 2). Time had a significant positive effect on the proportion of successful dispersers and males and

females were not significantly different from one another, consistent with the previous model. There was a significant negative effect of tubing angle on the proportion of successful dispersers suggesting that the more vertical tubing angle in Design 1 reduced dispersal success compared to Design 2, which had more horizontal tubing (Table 2).

Table 2 Generalised linear mixed effects model (GLMM) of the effect of time, sex, and tubing insertion angle on the proportion of successful dispersers from container *A* to *C* between Designs 1 and 2.

	Estimate	Std. Error	Z-value	P-value
Intercept	-3.339	0.388	-8.61	<0.001
Time (hours)	0.559	0.012	46.72	<0.001
Sex	0.338	0.223	1.52	0.129
Tubing angle	-0.642	0.299	-2.15	0.032

Random effect: replicate (intercept) variance = 0.282, std. dev. = 0.531

Discussion

Apparatuses to simulate long-distance dispersal in the laboratory using *Tribolium* beetles have been used for more than 50 years, however many of these studies have not specified apparatus design attributes. In particular, the slope and length of tubing between containers were expected to be critical to the movement of the individual beetles, as even shallow increases in the slope of a gradient can affect the distribution and orientation of *Tribolium* beetles (Graham & Waterhouse, 1964). This is consistent with our finding that increasing the steepness of the tubing angle between containers reduces dispersal success. The energy required for insects to climb vertically is much greater than that required to move laterally

(Full & Tullis, 1990), and as distance and time spent moving along a steep incline increases, the frequency of potential climbing errors increases. The slope of the tubing limited dispersal across designs with otherwise identical tubing length, but the difference between the proportion of beetles that successfully dispersed, for example in Design 1 and 4, were not different. While the effect of tubing insertion angle is significant, if tubing length is increased (i.e., to about 30 cm (Ritte & Lavie, 1977)), the effect of the steep climb may be offset.

The significant effect of tubing angle suggests that the near-vertical section of string that must be climbed prior to reaching the tubing also reduces dispersal rate. The string climbing ability of *T. castaneum* has not been explicitly tested, but this species can climb paper materials (Cline & Highland, 1976), readily climbs up string within an apparatus (Ogden, 1970b), attempts to climb the walls of housing (Ghent, 1963; Surtees, 1963), and is frequently seen climbing bag stacks and walls in storages (GH Walter, pers. obs.). Across all of the designs tested in the present study, a near-vertical portion of string (about 30 mm long) came immediately before the section of tubing where the angle could then be engaged by the climbing beetle. This section requires an ability to climb successfully upwards into the tubing, which plateaus, and then descends towards the next container. It seems likely that the near-vertical climb to the tubing would also constrain dispersal rate, and this may partially explain the exceptionally slow dispersal rate of *T. confusum* in the study by Łomnicki (2006), as the beakers used there had a vertical string section greater than 40 mm between the flour surface and the tubing. Therefore, the care needs to be taken to ensure that the near-vertical section of string is consistent in length, as it has a similar or perhaps stronger effect on dispersal rate than the tubing insertion angle. As *Tribolium* beetles are highly mobile animals, this design component is essential to constrain movement to sort dispersers from non-dispersers over a practical period of time.

While we found an overall significant decrease in the proportion of successful dispersers with increased tubing length, this effect was only apparent when comparing the shortest (140 mm) and the longest (640 mm) tubing lengths between containers. The long tubing used in Design 5 reduced the proportion of successful dispersers but was only significantly lower than Design 2. Additionally, the extreme length between containers of Design 5 made it impractical due to size. For the remaining tubing lengths between the containers (140–335 mm) dispersal rates were similar, which indicates that at least within a practical range, time spent within the apparatus tubing does not strongly limit dispersal.

In *Tribolium* beetles, dispersal is dependent on habitat deterioration, where increasing population density or reducing flour volume increases dispersal rate due to conditioning of the flour by frass accumulation, nutrient depletion and release of quinones by adults (Ogden, 1969; Zyromska-Rudzka, 1966a). Adult *T. castaneum* are strongly repelled by the smell of same-sex conspecifics, and this repulsion is enhanced when flour becomes "conditioned" (Ghent, 1963; Naylor, 1961; Ogden, 1970b). Thus, unmated individuals in a container with conspecifics will readily disperse as the flour becomes increasingly conditioned, and as finding mates becomes a priority. Therefore, the essentially linear increase in successful dispersers over time may reflect the decreasing population density as individuals emigrate and the continuous but ever-decreasing rate of flour conditioning as population density decreases.

We did not identify a significant difference in dispersal success between the sexes overall, but a greater proportion of males dispersed successfully in Design 2. Males were predicted to disperse faster than females, as previous studies have found males are more active or exploratory in dispersal apparatuses (Ogden, 1970b; Prus, 1966). However, Ziegler (1976)

found that dispersal rates were similar across males and females, as in the present study. We suggest that the absence of potential mates, and repulsion by the scent of same-sex individuals and conditioned flour drove dispersal at a similar rate in both sexes.

The present study demonstrates that tubing length and tubing insertion angle, which have been inconsistent among previous studies, can alter dispersal success for this species but not to the extent that dispersers cannot be effectively sorted from non-dispersers. This general apparatus appears to have relatively flexible design tolerances, and can achieve repeatable, controlled dispersal over replicate experiments. For the logistics of assessing dispersal ability of *T. castaneum*, which is highly active, restricting dispersal rate is important. We suggest that in addition to tubing length and insertion angle, the process of locating and climbing the vertical string section, and the inclusion of an intermediate container, reduces dispersal to a practical rate. In the present study, the time taken for more than 50% of individuals to successfully disperse across apparatus designs (about 70–85 h) would be feasible to experimentally separate dispersers from non-dispersers. Apparatus designs with a manageable tubing length and more horizontal tubing angle (i.e., Design 2 or 3) could be used to assess dispersal of other small insects that use pedestrian locomotion, including potential and current pest species. More than 50 years after its conception, the laboratory dispersal apparatus remains useful for assessing dispersal and addressing questions in microcosm-based species ecology.

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Data accessibility

Data will be deposited in the Dryad data repository upon acceptance for publication.

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